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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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R C van Dijk

DEN HAAG, DEN
THE HAGUE, 19/09/03
LA HAYE, LE

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Blatt 2 der Bescheinigung
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Demandeur(s):
Erasmus Universiteit Rotterdam
3015 GE Rotterdam
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Immunoregulator from pregnant mammals

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The invention relates to the field of immunology, more specifically to the field of immune-mediated disorders such as allergies, auto-immune disease, transplantation-related disease or inflammatory disease.

The immune system produces cytokines and other humoral factors to protect the host when threatened by inflammatory agents, microbial invasion, or injury. In most cases this complex defence network successfully restores normal homeostasis, but at other times the immunological mediators may actually prove deleterious to the host. Some examples of immune disease and immune system-mediated injury have been extensively investigated including anaphylactic shock, autoimmune disease, and immune complex disorders.

Recent advances in humoral and cellular immunology, molecular biology and pathology have influenced current thinking about auto-immunity being a component of immune-mediated disease. These advances have increased our understanding of the basic aspects of antibody, B-cell, and T-cell diversity, the generation of cellular and humoral immune responses and their interdependence, the mechanisms of (self)-tolerance induction and the means by which immunological reactivity develops against auto-antigenic constituents.

Since 1900, the central dogma of immunology has been that the immune system does not normally react to self. However, it as recently become apparent that auto-immune responses are not as rare as once thought and that not all auto-immune responses are harmful; some responses play a distinct role in mediating the immune response in general. For example, certain forms of auto-immune response such as recognition of cell surface antigens

encoded by the major histocompatibility complex (MHC) and of anti-idiotypic responses against self idiotypes are important, indeed essential, for the diversification and normal functioning of the intact immune system.

Apparently, an intricate system of checks and balances is maintained between various subsets of cells (i.e. T-cells) of the immune system, thereby providing the individual with an immune system capable of coping with foreign invaders. In that sense, auto-immunity plays a regulating role in the immune system.

However, it is now also recognised that an abnormal auto-immune response is sometimes a primary cause and at other times a secondary contributor to many human and animal diseases. Types of auto-immune disease frequently overlap, and more than one auto-immune disorder tends to occur in the same individual, especially in those with auto-immune endocrinopathies. Auto-immune syndromes may be mediated with lymphoid hyperplasia, malignant lymphocytic or plasma cell proliferation and immunodeficiency disorders such as hypogammaglobulinaemie, selective Ig deficiencies and complement component deficiencies.

Auto-immune diseases, such as systemic lupus erythematosus, diabetes, rheumatoid arthritis, post-partum thyroid dysfunction, auto-immune thromocytopenia, to name a few, are characterised by auto-immune responses, for example directed against widely distributed self-antigenic determinants, or directed against organ- or tissue specific antigens. Such disease may follow abnormal immune responses against only one antigenic target, ore against many self antigens. In many instances, it is not clear whether auto-immune responses are directed against unmodified self-antigens or self-antigens that have been modified (or resemble) any of numerous agents such as viruses, bacterial antigens and haptenic groups.

There is as yet no established unifying concept to explain the origin and pathogenesis of the various auto-immune disorders. Studies in experimental animals support the notion that auto-immune diseases may result from a wide spectrum of genetic and immunological abnormalities which differ from one individual to another and may express themselves early or late in life depending on the presence or absence of many superimposed exogenous (viruses, bacteria) or endogenous (hormones, cytokines, abnormal genes) accelerating factors.

It is evident that similar checks and balances that keep primary auto-immune disease at bay are also compromised in immune mediated disorders, such as allergy (asthma), acute inflammatory disease such as sepsis or septic shock, chronic inflammatory disease (i.e rheumatic disease, Sjögrens syndrome, multiple sclerosis), transplantation-related immune responses (graft-versus-host-disease, post-transfusion thrombocytopenia), and many others wherein the responsible antigens (at least initially) may not be self-antigens but wherein the immune response to said antigen is in principle not wanted and detrimental to the individual. Sepsis is a syndrome in which immune mediators, induced by for example microbial invasion, injury or through other factors, induce an acute state of inflammation which leads to abnormal homeostasis, organ damage and eventually to lethal shock. Sepsis refers to a systemic response to serious infection. Patients with sepsis usually manifest fever, tachycardia, tachypnea, leukocytosis, and a localized site of infection. Microbiologic cultures from blood or the infection site are frequently, though not invariably, positive. When this syndrome results in hypotension or multiple organ system failure (MOSF), the condition is called sepsis or septic shock. Initially, micro-organisms proliferate at a nidus of infection. The organisms may invade the

bloodstream, resulting in positive blood cultures, or might grow locally and release a variety of substances into the bloodstream. Such substances, when of pathogenic nature are grouped into two basic categories: endotoxins and exotoxins. Endotoxins typically consist of structural components of the micro-organisms, such as teichoic acid antigens from staphylococci or endotoxins from gram-negative organisms µlike LPS). Exotoxins (e.g., toxic shock syndrome toxin-1, or staphlococcal enterotoxin A, B or C) are synthesised and directly released by the micro-organisms.

As suggested by their name, both of these types of bacterial toxins have pathogenic effects, stimulating the release of a large number of endogenous host-derived immunological mediators from plasma protein precursors or cells (monocytes/macrophages, endothelial cells, neutrophils, T cells, and others).

It is in fact generally these immunological mediators which cause the tissue and organ damage associated with sepsis or septic shock. Some of these effects stem from direct mediator-induced injury to organs. However, a portion of shock-associated-organ dysfunction is probably due to mediator-induced abnormalities in vasculature, resulting in abnormalities of systemic and regional blood flow, causing refractory hypotension or MOSF (Bennett et al.).

The non-obese diabetic (NOD) mouse is a model for auto-immune disease, in this case insulin-dependent diabetes mellitus (IDDM) which main clinical feature is elevated blood glucose levels (hyperglycemia). Said elevated blood glucose level is caused by auto-immune destruction of insulin-producing ß cells in the islets of Langerhans of the pancreas (Bach et al. 1991, Atkinson et al. 1994). This is accompanied by a massive cellular infiltration surrounding and penetrating the islets (insulitis) composed of a heterogeneous mixture of CD4+

and CD8+ T lymphocytes, B lymphocytes, macrophages and dendritic cells (O'Reilly et al. 1991).

The NOD mouse represents a model in which auto-immunity against beta-cells is the primary event in the development of IDDM. Diabetogenesis is mediated through a multifactorial interaction between a unique MHC class II gene and multiple, unlinked, genetic loci, as in the human disease. Moreover, the NOD mouse demonstrates beautifully the critical interaction between heredity and environment, and between primary and secondary auto-immunity, its clinical manifestation is for example depending on various external conditions, most importantly of the micro-organism load of the environment in which the NOD mouse is housed.

As for auto-immunity demonstrable in NOD mice, most antigen-specific antibodies and T-cell responses are measured after these antigens were detected as self-antigens in diabetic patients. Understanding the role these auto-antigens play in NOD diabetes may further allow to distinguish between pathogenic auto-antigens and auto-immunity that is an epiphenomenon.

In general, T lymphocytes play a pivotal role in initiating the immune mediated disease process (Sempe et al. 1991, Miyazaki et al. 1985, Harada et al. 1986, Makino et al. 1986). CD4+ T-cells can be separated into at least two major subsets Th1 and Th2. Activated Th1 cells secrete IFN-γ and TNF-α, while Th2 cells produce IL-4, IL-5 and IL-10. Th1 cells are critically involved in the generation of effective cellular immunity, whereas Th2 cells are instrumental in the generation of humoral and mucosal immunity and allergy, including the activation of eosinophils and mast cells and the production of IgE (Abbas et al. 1996). A number of studies have now correlated diabetes in mice and human with Th1 phenotype development (Liblau et al. 1995, Katz et al. 1995). On the other hand, Th2 T cells are shown to

be relatively innocuous. Some have even speculated that Th2 T cells in fact, may be protective. Katz et al. have shown that the ability of CD4+ T cells to transfer diabetes to naïve recipients resided not with the antigen specificity recognised by the TCR per se, but with the phenotypic nature of the T cell response. Strongly polarised Th1 T cells transferred disease into NOD neonatal mice, while Th2 T cells did not, despite being activated and bearing the same TCR as the diabetogenic Th1 T cell population. Moreover, upon co-transfer, Th2 T cells could not ameliorate the Th1-induced diabetes, even when Th2 cells were co-transferred in 10-fold excess (Pakala et al. 1997).

The incidence of sepsis or septic shock has been increasing since the 1930's, and all recent evidence suggests that this rise will continue. The reasons for this increasing incidence are many: increased use of invasive devices such as intravascular catheters, widespread use of cytotoxic and immunosuppressive drug therapies for cancer and transplantation, increased longevity of patients with cancer and diabetes who are prone to develop sepsis, and an increase in infections due to antibiotic-resistant organisms. Sepsis or septic shock is the most common cause of death in intensive care units, and it is the thirteenth most common cause of death in the United States. The precise incidence of the disease is not known because it is not reportable; however, a reasonable annual estimate for the United States is 400,000 bouts of sepsis, 200,000 cases of septic shock, and 100,000 deaths from this disease.

Various micro-organisms, such as Gram-negative and Gram-positive bacteria, as well as fungi, can cause sepsis and septic shock. Certain viruses and rickettsiae probably can produce a similar syndrome. Compared with Gram-positive organisms, Gram-negative bacteria are somewhat more likely to produce sepsis or septic shock.

Any site of infection can result in sepsis or septic shock. Frequent causes of sepsis are pyelonephritis, pneumonia, peritonitis, cholangitis, cellulitis, or meningitis. Many of these infections are nosocomial, occurring in patients hospitalised for other medical problems. In patients with normal host defences, a site of infection is identified in most patients. However, in neutropenic patients, a clinical infection site is found in less than half of septic patients, probably because small, clinically inapparent infectious in skin or bowel can lead to bloodstream invasion in the absence of adequate circulating neutrophils. Clearly there is a need to protect against sepsis or septic shock in patients running such risks.

Recently, considerable effort has been directed toward identifying septic patients early in their clinical course, when therapies are most likely to be effective. Definitions have incorporated manifestations of the systemic response to infection (fever, tachycardia, tachypnea, and leukocytosis) along with evidence of organ system dysfunction (cardiovascular, respiratory, renal, hepatic, central nervous system, hematologic, or metabolic abnormalities). The most recent definitions use the term systemic inflammatory response syndrome (SIRS) emphasising that sepsis is one example of the body's immunologically-mediated inflammatory responses that can be triggered not only by infections but also by noninfectious disorders, such as trauma and pancreatitis (for interrelationships among systemic inflammatory response (SIRS), sepsis, and infection, see Crit. Care Med. 20:864, 1992; For a review of pathogenic sequences of the events in sepsis or septic shock see N Engl J Med 328:1471, 1993).

Toxic shock syndrome toxin (TSST-1) represents the most clinically relevant exotoxin, identified as being the causative agent in over 90% of toxic shock syndrome

cases (where toxic shock is defined as sepsis or septic shock caused by super-antigenic exotoxins). Super antigens differ from "regular" antigens in that they require no cellular processing before being displayed on a MHC molecule. Instead they bind to a semi-conserved region on the exterior of the TCR and cause false "recognition" of self antigens displayed on MHC class II (Perkins et al.; Huber et al. 1993). This results in "false" activation of both the T cell and APC leading to proliferation, activation of effector functions and cytokine secretion. Due to the superantigen's polyclonal activation of T cells, a systemic wide shock results due to excessive inflammatory cytokine release. (Huber et al. 1993, Miethke et al. 1992).

The inflammatory cytokines involved in sepsis are similar. These immunological mediators are tumor necrosis factor (TNF), interferon gamma (IFN-gamma), nitric oxide (Nox) and interleukin 1(IL-1), which are massively released by monocytes, macrophages and other leukocytes in response to bacterial toxins (Bennett et al., Gutierrez-Ramos et al 1997). The release of TNF and other endogenous mediators may lead to several patho-physiological reactions in sepsis, such as fever, leukopenia, thrombocytopenia, hemodynamic changes, disseminated intravascular coagulation, as well as leukocyte infiltration and inflammation in various organs, all of which may ultimately lead to death. TNF also causes endothelial cells to express adhesion receptors (selectins) and can activate neutrophils to express ligands for these receptors which help neutrophils to adhere with endothelial cell surface for adherence, margination, and migration into tissue inflammatory foci (Bennett et al.). Blocking the adhesion process with monoclonal antibodies prevents tissue injury and improves survival in certain animal models of sepsis or septic shock (Bennett et al.).

These findings, both with auto-immune disease, as well as with acute and chronic inflammatory disease, underwrite the postulated existence of cells regulating the balance between activated Th-sub-populations. Possible disturbances in this balance that are induced by altered reactivity of such regulatory T cell populations can cause immune-mediated diseases, which results in absence or over-production of certain critically important cytokines (O'Garra et al. 1997). These Th-sub-populations are potential targets for pharmacological regulation of immune responses.

In general, immune mediated disorders are difficult to treat. Often, broad-acting medication is applied, such as treatment with corticosteroids or any other broad acting anti-inflammatory agent that in many aspects may be detrimental to a treated individual.

In general there is a need for better and more specific possibilities to regulate the checks and balances of the immune system and treat immune mediated disorders.

The invention provides a pharmaceutical composition for treating an immune-mediated disorder such as an allergy, auto-immune disease, transplantation-related disease or acute or chronic inflammatory disease and/or provides an immunoregulator (IR), for example for stimulating or regulating lymphocyte action comprising an active component having an apparent molecular weight of 58 to 15 kilodalton as determined in gel-permeation chromatography, said active component capable of stimulating splenocytes obtained from a 20-week-old female non-obese diabetes (NOD) mouse, said stimulated splenocytes delaying the onset of diabetes in a NOD-severe-combined-immunodeficient (NOD.scid) mouse reconstituted at 8 weeks old with said splenocytes, or

comprising an active component functionally related thereto.

In one embodiment, the invention provides an pharmaceutical composition or immunoregulator wherein said active component is capable of inhibiting gamma-interferon production or stimulating interleukine-4 production of splenocytes obtained from a 20-week-old female non-obese diabetes (NOD) mouse. Preferably, said composition or immunoregulator is obtainable from a pregnant mammal, preferably a human, for example obtainable from a pharmacological preparation prepared to contain (placental) gonadotropins such as pregnant mare serum gonadotropin (PMSG) found in serum of pregnant mares (IR-S), or pregnant mouse uterus extract (PMUE) extracted from uteri (IR-UE) of gravid mice or human chorionic gonadotropin (hCG or HCG) found in blood or urine of pregnant women.

Clinical grade preparations of gonadotropins such as hCG and PMSG have since long been used to help treat reproductive failure in situations where follicular growth or stimulation of ovulation is desired. Said preparations are generally obtained from serum or urine, and often vary in degree of purification and relative activity, depending on initial concentration in serum or urine and depending on the various methods of preparation used.

In a particular embodiment, the invention provides a immunoregulator comprising an active component obtainable from a mammalian CG preparation present in a fraction which elutes with an apparent molecular weight of 58 to 15 kilodalton as determined in gel-permeation chromatography, said active component capable of stimulating splenocytes obtained from a non-obese diabetes (NOD) mouse, or comprising an active component functionally related to said active compound, for example

wherein said stimulated splenocytes are capable of delaying the onset of diabetes in a NOD-severe-combined-immunodeficient mouse reconstituted with said splenocytes.

The invention also provides an immunoregulator wherein said active component is capable of inhibiting gamma-interferon production obtained from a non-obese diabetes (NOD) mouse. The invention also provides an immunoregulator wherein said active component is capable of stimulating interleukine-4 production of splenocytes obtained from a non-obese diabetes (NOD) mouse.

An immunoregulator as provided by the invention (IR) with or without hCG as for example present in the urine of first trimester of pregnancy (IR-U) and in commercial hCG preparations (IR-P) has immune regulatory effects. In particular, IR can inhibit or regulate auto-immune and acute- and chronic-inflammatory diseases. TNF and IFN-gamma are pathologically involved in acute inflammatory disease such as sepsis or septic shock and also in auto-immune and chronic inflammatory diseases. Since IR has the ability to regulate T-cell sub-populations and inhibit TNF and IFN-gamma, IR can be used to treat, suppress or prevent immune mediator disorders such as sepsis or septic shock (acute inflammatory disease) as well as auto-immune disease or chronic inflammatory diseases such as systemic lupus erythematosus, diabetes, rheumatoid arthritis, post-partum thyroid dysfunction, auto-immune thromocytopenia and others, such as allergies and chronic inflammatory disease (i.e. rheumatic disease, Sjögrens syndrome, multiple sclerosis)and transplantation related immune responses. Our results for example show that IR inhibit sepsis or septic shock caused by endotoxin or by exotoxin. IR as provided by the invention inhibits or counters immune mediated auto-immune diseases, chronic inflammatory diseases as well as acute inflammatory diseases.

Anecdotal observations and laboratory studies indicated previously that hCG might have an anti-Kaposi's sarcoma and anti-human-immunodeficiency-virus effect (Treatment Issues, July/August 1995, page 15. It has been observed that hCG preparations have a direct apoptotic (cytotoxic) effect on Kaposi's sarcoma (KS) in vitro and in immunodeficient patients and mice and a prohematopoetic effect on immunodeficient patients (Lunardi-Iskandar et al., Nature 375, 64-68; Gill et al., New. Eng. J. Med. 335, 1261-1269, 1996; US patent 5677275), and a direct inhibitory antiviral effect on human and simian immunodeficiency virus (HIV and SIV) (Lunardi-Iskandar et al., Nature Med. 4, 428-434, 1998, US patent 5700781). Said cytotoxic and anti-viral effects have also been attributed to an unknown hCG mediated factor (HAF), present in clinical grade preparations of hCG. However, commercial hCG preparations (such as CG-10, Steris Profasi, Pregnyl, Choragon, Serono Profasi, APL), have various effects. Analysis of several of these, (AIDS, 11: 1333-1340, 1997) for example shows that only some (such as CG-10, Steris Profasi) are KS-killing whereas others (Pregnyl, Choragon, Serono Profasi) were not. Secondly, recombinant subunits of (α or β) hCG were killing but intact recombinant hCH not. It was also found that the killing effect was also seen with lymphocytes. Therapy of KS has recently been directed at using beta-hCG for its anti-tumour effect Eur. J. Med Res. 21: 155-158, 1997, and it was reported that the beta-core fragment isolated from urine had the highest apoptotic activity on KS cells (AIDS, 11: ,713-721, 1997).

The invention provides an immunoregulator or a pharmaceutical composition for treating an immune-mediated disorder obtainable from a hCG preparation or a fraction derived thereof. The effects of said immunoregulator include a stimulating effect on lymphocyte populations (such as found in peripheral

lymphocytes, thymocytes or splenocytes), instead of cytotoxic or anti-viral effects. The invention provides a method for treating an immune-mediated-disorder comprising subjecting an animal to treatment with at least one immunoregulator obtainable from a pregnant mammal. Said treatment can be direct, for example treatment can comprise providing said individual with a pharmaceutical composition, such as a hCG or PMSG preparation, comprising an immunoregulator as provided by the invention. It is also possible to provide said pharmaceutical composition with a fraction or fractions derived from a pregnant animal by for example sampling urine or serum or placental (be it of maternal or foetal origin) or other tissue or cells and preparing said immunoregulator comprising said active component from said urine or serum or tissue or cells by fractionation techniques known in the art (for example by gel permeation chromatograpy) and testing for its active component by stimulating a NOD mouse or its splenocytes as described.

The invention also provides a method for in vitro stimulation of lymphocytes and transferring said stimulated lymphocytes as a pharmaceutical composition to an animal for treating said animal for an immune mediated disorder. In a particular embodiment of the invention a pharmaceutical composition is provided comprising lymphocytes stimulated in vitro with an immunoregulator provided by the invention.

In a preferred embodiment of the invention, said disorder comprises diabetes, yet other immune mediated disorders, such as acute and chronic inflammation, can also be treated. In yet another preferred embodiment, said disorder comprises sepsis or septic shock. The invention provides a method of treatment for an animal, preferably wherein said animal is human.

In a particular embodiment, a method provided by the invention is further comprising regulating relative ratios and /or cytokine activity or cytokine expression or marker expression of lymphocyte subset-populations in said animal, such as subset-populations that comprise Th1 or Th2 cells, or Th3 or Th8 cells, or other effector or regulatory T-cell populations.

The invention also provides an immunoregulator for use in a method according to the invention, and use of said immunoregulator, preferably obtainable from a pregnant mammal, for the production of a pharmaceutical composition for the treatment of an immune-mediated-disorder, preferably selected from a group consisting of allergies, auto-immune disease, transplantation-related disease and acute and chronic inflammatory disease. In a particular embodiment said immunoregulator comprises a clinical grade hCG or PMSG preparation or a fraction derived thereof. For example, the invention provides use of a hCG preparation, or a preparation functionally equivalent thereto, for the preparation of a pharmaceutical composition for the treatment of diabetes. In yet another example, the invention provides use of a hCG preparation, or a preparation functionally equivalent thereto, for the preparation of a pharmaceutical composition for the treatment or prevention of sepsis or septic shock.

The invention is further explained in the experimental part without limiting the invention thereto.

Experimental part

Immunoregulator (IR)

hCG fraction preparation and characterisation. Gel filtration of the solution of 1 or 2 vials of commercial grade hCG-Pregnyl (5,000 IU/vial) was performed on a

Pharmacia FPLC sytem equipted with a Superdex 75 column (HR 5/30) (Pharmacia, Sweden) in PBS. Sample load volume was 1 ml. The flow rate was 0.5 ml/min for 45 min followed. The 1 minute flow rate of 0.2 ml/min was implemented because of the viscosity of the commercial grade hCG solution which has a high lactose content. hCG and a very low amount hCG core fragment were present in the relatively purified Pregnyl preparation of hCG and their positions were used as internal size markers. hCG eluted as 78kDa molecule and the hCG β-core eluted as a 19 kDa molecules on gel filtration. There were 1-5 fractions collected whereby fraction 1-2 contained hCG and fraction 5 contained the hCG (-core fragments. Fraction 1-2 and fraction 3-5 were tested for anti-diabetic effect by treating in vitro total spleen cells of 20-wk-old NOD and transferring them into NOD.scid. In this way human recombinant hCG, α-hCG, and β-hCG (Sigma, St. Louis, MO. USA) were also tested.

IR-U purification from first trimester pregnancy urine: First trimester pregnancy urine (2 litres) was collected in a bottle from a healthy volunteer and was refrigerated until delivered at the laboratory within 2 days. Upon delivery, 1 gram per litre of sodium azide was added and the pH was adjusted to 7.2-7.4 with sodium hydroxide and allowed to sediment for 1 hour (h) at room temperature (RT). Approximately, 75% of the supernatant was decanted and the remainder close to the precipitate was centrifuged (10 min at 25000 rpm at 40C) to remove sediment and added to the rest of the supernatants. The supernatants was filtered through 0.45 (m in a Minitan (Millipore) transversal filtration set-up. Subsequently, the filtrate (2 litre) was concentrated in an Amicon ultrafiltration set-up equipped with an YM Diopore membrane with a 10 kDa cut-off. The final volume (250 ml) was dialysed against 2 changes of 10 litres of Milli Q

water. Next the sample was further concentrated by 10 kDa cut-off in an Amicon ultrafiltration to a final volume of 3 ml.

Gel permeation: A Pharmacia FPLC system equipped with a Superdex 75 gel permeation column was used to analyse the treated urine sample (IR-U) and commercial hCG preparation (IR-P) (Pregnyl; Organon; Oss, NL). The running conditions used are shown below:

```
0.0  CONC %B    0.0
0.0  ML/MIN     0.20
0.5  ML/MIN     0.50
0.5  CM/ML      0.50
0.8  ML/MIN     1.00
0.8  CM/ML      1.00
2.0  CLEAR DATA
     HOLD
2.0  VALVE.POS  1.2
2.0  MONITOR    1
2.0  LEVEL %    5.0
2.0  ML/MARK    2.0
2.0  INTEGRATE  1
4.0  VALVE.POS  1.1
6.0  PORT.SET   6.1
50.0 INTEGRATE  0
52.0 CONC %B    0.0
```

Anion exchange chromatography: In order to further separate the overlapping fractions, 1 ml MONO Q HR 5/5 FPLC anion exchange column was used. The running conditions are shown below and the buffer combination consisted of 10mM PBS, pH 7.3 as buffer A and PBS containing 1 M NaCl as buffer B:

```
0.0  CONC %B    0.0
```

```
0.0  ML/MIN      1.00
0.0  CM/ML       1.00
1.0  ALARM       0.1
1.0  HOLD
1.0  CLEAR DATA
1.0  MONITOR     1
1.0  LEVEL %     5.0
1.0  ML/MARK     2.0
1.0  INTEGRATE   1
1.0  PORT.SET    6.0
1.0  VALVE.POS   1.2
6.0  CONC %B     0.0
6.0  PORT.SET    6.0
11.0 CONC %B     50.0
14.0 CONC %B     50.0
16.0 CONC %B     100
16.0 PORT.SET    6.0
18.0 CONC %B     100
18.0 CONC %B     0.0
18.0 INTEGRATE   0
25.0 CONC %B     0.0
```

Further treatment of the IR-U and IR-P: To reduce covalent binding between protein species present in the urine sample, we treated the urine (IR-U) and hCG preparation (IR-P) sample with 60 mM 2-mercaptoethanol for 3 min at 100 0C. Subsequently, the treated IR-U and IR-P sample were applied to the Superdex 75 column under identical running conditions.

Activity determination of FPLC fractions of IR-U: The protein concentration of urine fractions was determined by OD280 nm divided by 1.4. From this value, the amount of hCG units was calculated using 5000 IU/ml Pregnyl preparation of hCG corresponded to 100 µg.

Alternative methods for purifying and/or isolating IR comprise gelfiltration on for example a Superdex 75 column in a FPLC system using PBS with or without ethanol to increase resolution and disrupt hydrophobic interactions, optionally followed by cationic exchange. Samples can be submitted in reduced or unreduced form. Another method comprises lectin affinity chromatography to better separate carbohydrate containing components from other components, whereby the effluent is further subjected to gel filtration. It is of course possible to derive at synthetic or recombinant (poly)peptide sequences with methods known in the art, and to select (synthetic) antibodies, i.e. phage-derived, to further select IR.

Auto-immune disease experiments

The non-obese diabetic (NOD) mouse is a model for auto-immune disease, in this case insulin-dependent diabetes mellitus (IDDM), which main clinical feature is elevated blood glucose levels (hyperglycemia). The elevated blood glucose levels are caused by the immune-mediated destruction of insulin-producing ß cells in the islets of Langerhans of the pancreas (Bach et al. 1991, Atkinson et al. 1994). This destruction is accompanied by a massive cellular infiltration surrounding and penetrating of the islets (insulitis) by a heterogeneous mixture composed of a CD4+ and CD8+ T lymphocytes, B lymphocytes, macrophages and dendritic cells (O'Reilly et al. 1991). The easiest and most reliable way to detect the onset of diabetes in these mice is to test for glucose levels in the blood.

The NOD mouse represents a model in which auto-immunity against beta-cells is the primary event in the development of IDDM. In general, T lymphocytes play a

pivotal role in initiating the disease process (Sempe et al. 1991, Miyazaki et al. 1985, Harada et al. 1986, Makino et al. 1986). Diabetogenesis is mediated through a multifactorial interaction between a unique MHC class II gene and multiple, unlinked, genetic loci as in the human disease. Moreover, the NOD mouse demonstrates beautifully the critical interaction between heredity and environment. Differences between the cleanliness of the housing conditions illustrates how environmental factors can effect the action of diabetes-mediated genes (Elias et al. 1994).

As for the auto-immunity recorded in NOD mice, most antigen-specific antibodies and T-cell responses have been studied after these antigens were detected as self-antigens in diabetic patients. Understanding the role that these auto-antigens play in NOD diabetes may allow to distinguish between primary pathogenic auto-antigens and auto-immunity that is an epiphenomenon. Moreover, one should bear in mind that IDDM patients are genetically and pathogenically heterogeneous.

A typical longitudinal histological examination of the NOD pancreas demonstrates infiltrating cells surrounding the blood vessels at 3-4 weeks of age, but the islets are typically still clear at 6-7 weeks. Infiltrating cells than reach the islets, either surrounding them or accumulating at one pole. Between 10 and 12 weeks, the infiltrating cells penetrate into the islets and the islets become swollen with lymphocytes. As mentioned above, differences between the housing conditions and microbiological and environmental factors can effect the penetrance of diabetes-susceptible genes.

In our hands, typically between 14-17 weeks NOD mice become diabetic. However, this varies from lab to lab (average 14-19 weeks) (Elias et al. 1994).

CD4+ T-cells can be separated into at least two major subsets Th1 and Th2. Activated Th1 cells secrete IFN-γ and

TNF-α, while Th2 cells produce IL-4, IL-5 and IL-10. Th1 cells are critically involved in the generation of effective cellular immunity, whereas Th2 cells are instrumental in the generation of humoral and mucosal immunity and allergy, including the activation of eosinophils and mast cells and the production of IgE (Abbas et al. 1996). A number of studies have now correlated diabetes in mice and human with Th1 phenotype development (Liblau et al. 1995, Katz et al. 1995).

Th2 T cells are shown to be relatively innocuous. Some have even speculated that Th2 T cells in fact, may be protective. But Katz et al. have shown the ability of CD4+ T cells to transfer diabetes to naïve recipients resided not with the antigen specificity recognised by the TCR, per se, but with the phenotypic nature of the T cell response. Strongly polarised Th1 T cells transferred disease into NOD neonatal mice, while Th2 T cells did not, despite being activated and bearing the same TCR as the diabetogenic Th1 T cell population. Moreover, upon co-transfer, Th2 T cells could not ameliorate Th1-induced diabetes, even when Th2 cells were co-transferred in 10-fold excess (Pakala et al. 1997).

Th1-polarized T cells can transfer disease in neonatal NOD mice, something Th2-polarized T cells fail to do, both Th1- and Th2-polarized T cells can transfer disease in NOD.scid mice and other immune-compromised recipients. Th2-mediated diabetes in NOD.scid recipients exhibited a longer pre-diabetic phase and a lowered overall incidence. Moreover, the diabetic lesion created by Th2 cells is unique and quite unlike the lesion found in spontaneously diabetic or Th1 T cell-induced diabetes in either neonates or NOD.scid mice (Pakala et al. 1997).

In addition, IFN-γ correlates with diabetes (in NOD as well as in humans) and anti-IFN-γ prevents disease; under disease IFN-γ+ cells are present in islets and antigen-

specific Th1 clones accelerate the onset of diabetes (Pakala et al. 1997, O'Garra et al. 1997). Furthermore, Th2 cells only induce insulitis in neonatal NOD, but have the capacity to induce diabetes in immuno-compromised NOD.scid; also, disease is inhibitable by anti-IL-10, but not by anti-IL-4 (Pakala et al. 1997). This suggests that non-Th2 type regulator T cells are present in normal mice, but these are absent in immunodeficient mice. These results stress the existence of cells regulating the balance between activated Th-sub-populations. Possible disturbances in this balance induced by altered reactivity of such regulatory T cell populations can cause immune-mediated diseases, which results in absence or over-production of certain critically important cytokines (O'Garra et al. 1997).

Recently, Gallo et. al. reported anti-Kaposi's Sarcoma, anti-HIV, anti-SIV and distinct hematopoietic effects of clinical grade crude preparations of human chorionic gonadotropin (hCG) (Lunardi-Iskandar et al. 1995, Gill et al. 1996, Lunardi-Iskandar et al. 1998). In contrast to their previous studies, it is also claimed that the anti-tumour and anti-viral activity of hCG preparation is not due to the native hCG heterodimer, including its purified subunits or its major degradation product, the ß-core; instead the active moiety resides in an as yet unidentified hCG mediated factor (HAF). Whatever the true factor may be, these unidentified factors in several hCG preparations have anti-tumour activity through the selective induction of apoptosis, besides direct cytotoxic effects on the tumour cells. Furthermore, they postulated that the anti-tumour activity could not be due to an immune-mediated response, since there was no infiltration of the tumour with mononuclear cells.

Moreover, the reported pro-hematopoietic effect of clinical grade hCG was noted in clinical studies in humans infected with HIV, (Lunardi-Iskandar et al. 1998)

indicating that the hematopoietic effect is indirect, and caused by rescuing CD4+ cells otherwise killed by HIV through the anti-HIV activity of hCG.

Some auto-immune diseases, in particular Th1 mediated diseases, like rheumatoid arthritis (RA) (Grossman et al. 1997, Russel et al. 1997, Buyon et al. 1998, Hintzen et al. 1997) can remit during pregnancy. Furthermore, successful pregnancy is a Th2 type phenomenon (Raghupath et al. 1997). We tested hCG preparation and its fractions from Pregnyl [Organon, Oss] on the development of diabetes in NOD mice and in a in vitro model.

Surprisingly, we found that intraperitoneal treatment of NOD mice of age 15 weeks, with a hCG preparation for three times a week for a month can delay or inhibit the onset of diabetes. In addition, transfer of total spleen cells from these treated NOD mice into NOD.scid mice can delay or prevent diabetes in NOD.scid whereas transfer of non-treated spleen cells cannot. This anti-diabetic effect resides in a fraction obtainable from pregnant woman but not in hCG.

Mice. NOD mice were bred in our facilities under specific pathogen-free conditions. The spontaneous incidence of diabetes in our colony is 85% in females at 15 weeks of age. NOD.scid mice were also bred in our facilities under specific pathogen-free conditions. Transfer of diabetogenic cells from NOD to NOD.scid at the age of 8 weeks induces diabetes after 22 days.

Diabetes. Diabetes was assessed by measurement of venous blood using an Abbott Medisense Precision Q.I.D. glucometer and also monitored for glucosuria (Gluketur Test; Boehringer Mannheim, Mannheim, Germany). Animals were considered diabetic after two consecutive glucose measurements of higher than 13.75 mmol/l (250 mg/dl).

Onset of diabetes was dated from the first consecutive reading. In instances of sustained hyperglycemia of >33 mmol/l animals were killed to avoid prolonged discomfort.

Immunohistochemistry. Mice were killed by CO2 asphyxiation. The entire pancreata were removed and snap frozen in OCT compound (Tissue-tek) for cry-sectioning. 5-µm cryo-sections were obtained, air dried, and stored at -20°C until used. Formalin-fixed sections were deparaffinised in xylene and alcohol, and stained with hematoxylin and eosin for general morphology. Immunohistochemistry for insulin was then performed using a two-step protocol. Endogenous peroxidase activity was blocked, and slides were incubated with a rabbit antiserum to insulin (Dako Corp., Carpenteria, CA; 1:500 in 5% normal mouse serum for 30 min). After washing steps, staining was revealed with horseradish peroxidase-conjugated anti-rabbit Ig (Dako; 1:500 in 5% NMS for 30 min), developed with amino-ethyl-carbazole (AEC; Pierce) for 10 min and mounted in crystalmount.

In vivo anti-diabetic effect: NOD mice at the age of 15 weeks were treated with PBS (n=4), 300 IU Pregnyl (n=4), or 600 IU Pregnyl (n=4) i.p., 3 times a week for four weeks and diabetes was assessed as mentioned above. After four weeks the treatment was stopped and the PBS and the 600 IU Pregnyl group were killed after one week. The 300 IU Pregnyl group was left alive till the age of 28 weeks. Spleen cell transfer. The spleen was removed from 600 IU Pregnyl treated NOD and PBS control treated NOD mice, and total spleen cells were recovered. These cells were washed twice with PBS and 20 x 10^6 cells were i.p. transferred into a 8-wk-old NOD.scid mouse.

In vitro restimulation. Total spleen cells (1 x 10^6 cells/ml) from 20-wk-old NOD were stimulated in RPMI+

suplemented with 10% FBS with LPS (Ecoli;10 μg/ml) or coated anti-CD3 (145-2c11;25 μg/ml) with different doses of hCG-Pregnyl (50, 100, 300, 600, 800 IU/ml), Fraction 1-2 (200 μg/ml), Fraction 3-5 (200(g/ml), human recombinant hCG, α-hCG, and β-hCG (each at 200 μg/ml) in flat bottom 96-well plates. Wells with anti-CD3 coating were implemented with IL-2 (40 IU/ml). Plates were incubated at 37°C in 5% CO2 in air for 48hrs. After 48hrs of incubation the supernatants were collected for cytokine analyses.

CD4+ T-cells were isolated from total spleen cells of 20-wk-old NOD and stimulated as mentioned above with anti-CD3 at different conditions. These wells were implemented with IL-2 (40 μg/ml) and anti-CD28 (10 μg/ml). After 48hrs of incubation the supernatants were also collected for cytokine analyses.

Cytokine ELISA. IL-4 was detected using monoclonal anti-IL-4 antibody (11B11) as the capture antibody and revealed with biotinated-conjugated rat anti-mouse IL-4 monoclonal antibody (BVD6 24G2.3). IFN-γ was detected using monoclonal anti-IFN-γ antibody (XMG1.2) as the capture antibody and revealed with biotinylated-conjugated rat anti-mouse IFN-γ monclonal antibody (R46A2). In both cases ABTS substrate was used for detection.

Sepsis or septic shock experiments.

There are three common mouse models used to investigate sepsis or septic shock: high dose LPS, low dose LPS with D-Galactosamine sensitisation and low dose superantigen with D-Galactosamine.

One of the first models used for investigating sepsis or septic shock involved treatments with rather

large doses of LPS in the inter-peritoneal cavity (between 300-1200μg). Mice are quite resistant to bacterial toxins, yet succumb to this high dose. It has been suggested that a high dose of LPS in mice might correlate with a lower dose in humans (Mietheke et al.) Approximately 70% of sepsis or septic shocks in humans are caused by Gram-negative bacterial endotoxin and up to 30% are created by exotoxins released from Gram-positive bacteria. The traditional endotoxin- the distinctive lipopolysaccharide (LPS) is associated with the cell membrane of the Gram-negative organism represents the most common initiator of the sepsis or septic shock pathogenetic cascade. The endotoxin molecule consists of an outer core with a series of oligosaccharides that are antigenically and structurally diverse, an inner oligosaccharide core that has similarities among common gram-negative bacteria, and a core lipid A that is highly conserved across bacterial species. The lipid A is responsible for many of the toxic properties of endotoxin. The systemic effects of endotoxins, such as LPS seem to be largely mediated by macrophages, since adoptive transfer of endotoxin-sensitive macrophages renders previously endotoxin resistant mice sensitive to the toxin (Freudenberg et al. 1986).

The more commonly used model of endotoxin sepsis or septic shock takes advantage of the increased susceptibility of BALB/c mice to low doses of LPS after being simultaneously treated with Galactosamine (D-Gal sensitized). This D-Gal treatment dramatically sensitizes animals to the toxic effect of LPS, so that nanogram amounts induce a liver toxicity that is lethal for wild-type animals in a period of 6-7 h. This systemic effects of endotoxin seem to be largely mediated by macrophages. (Gutierrez-Ramos et al. 1997). Although certain mediators are undoubtedly more important than other in producing sepsis, probably dozens of organism-

and host-derived mediators interacting, accelerating, and inhibiting one another, are responsible for the pathogenesis of sepsis or septic shock.

On response to LPS, TNF, and other mediators, endothelial cells and macrophages can release a potent vasodilator agent, endothelial-derived relaxing factor (EDRF), which has recently been identified as nitric oxide. This molecule causes smooth muscle cell relaxation and potent vasodilatation. Inhibiting nitric oxide production with competitive inhibitors of nitric oxide synthase results in increased blood pressure in animals with endotoxin shock. This suggests that nitric oxide may be partially responsible for the hypotension associated with sepsis. Although inhibition of nitric oxide restores blood pressure, such inhibition may reduce tissue blood flow. (Bennett et al.).

Endotoxin can also activate the complement cascade, usually via the alternative pathway. This results in the release of the anaphylotoxins C3a and C5a, which can induce vasodilatation, increased vascular permeability, platelet aggregation, activation and aggregation of neutrophils. These complement-derived mediators may be responsible in part for the microvascular abnormalities associated with sepsis or septic shock. Further, endotoxin can result in the release of bradykinin via the activation of Factor XII (Hageman factor), kallikrein, and kiniogen. Brandyinin is also a potent vasodilator and hypotensive agent. LPS activation of factor XII also leads to intrinsic and (through macrophage and endothelial cell release of tissue factor) extrinsic coagulation pathway activation. This result in consumption of coagulation factors and DIC. TNF also activates the extrinsic pathway and may contribute to these coagulation abnormalities.

Different metabolism of the arachidonic acid cascade are also known to cause vasodilatation (prostacyclins),

vasoconstriction (thromboxanes), platelet aggregation, or neutrophil activation. In experimental animals, inhibiting cyclo-oxygenase or thromboxane synthase has protected against endotoxin shock. Elevated levels of thromboxane B2 (TBX2) and 6-ketoprostaglandin F1 (the end product of prostacylin metabolism) are present in patients with sepsis. A number of cytokines can cause release of these arachidonic acid metabolites from endothelial cells or leukocytes.

In a similar fashion, exotoxin shock model D-Gal sensitised BALB/c mice are treated with low doses of TSST-1 or SEB. These superantigens stimulate the proliferation and activation of a large proportion of T cells. In fact, the T cell activation induced by these super-antigens can almost be viewed as a polyclonal T-cell activation in that T-cells expressing a specific V-beta family are all activated through non antigen specific binding of the TCR/MHCII/ and superantigen (Figure 14).

D-Galactosamine has been shown to be a transcription inhibitor which targets the liver, interfering with the synthesis of acute phase proteins. It is believed that these acute phase proteins infact help the liver detoxify/ or deactivate TNFα. In fact D-Galactosamine treatment in the low dose endotoxin or exotoxin models is acompanied by TNFα mediated hepatic apoptosis. D-galactosmine treatment alone does not result in hepatic apoptosis, and these organ damaging effects can be neutralised in both low dose models by neutralising anti-TNFα antibodies (Gutierrez-Ramos et al. 1997).

Mice used in sepsis or septic shock experiments: Female BALB/c and SJL mice between 8-12 weeks of age were used for all experiments. The animals were bred in our facility under specific pathogen-free conditions according to the protocols described in the Report of

European Laboratory Animal Science Associations (FELASA) Working group on Animal Health (Laboratory Animals 28: 1-24, 1994).

Injection Protocols: Toxic Shock (TSST-1 & D-Galactosamine) (n=6).

For the exotoxin model, Balb/c mice were injected with 20mg D-Galactosamine dissolved in 100 µl sterile saline solution (9%) intraperitoneally. They were then given 4µg of TSST-1 dissolved in 100µl sterile saline solution (9%) injected subcutaneously in two sites approximately .5cm below each shoulder blade. Control groups were injected with either 4µg TSST-1 subcutaneously without D-Galactosamine, or treated with D-Galactosamine alone. A group of D-Galactosamine sensitised Balb/c mice were also pre-treated i.p. with 700 IU IR-P for 3 days before the treatment of TSST-1.

LPS model (n=6)

For the endotoxin model, Balb/c and SJL mice were treated i.p. with 600 µg LPS. Control group were treated only with PBS i.p. To test the effect of IR-P, we also pretreated Balb/c and SJL mice with 700 IU for 3 days and then injected with 600 µg of LPS. Moreover, a group of Balb/c mice was also pretreated with IR-U fractions (IR-U1, IR-U2, IR-U3-5), each with same doses of 200 µg i.p. for 3 days and then injected with 600 µg of LPS.

We also treated Balb/c mice with 700 IU IR-P twice i.p. after 1 and 2 hours of injection with LPS respectively.

Semi-Quantitative Sickness Measurements: Mice were scored for sickness levels using the following measurement scheme:

1 Percolated fur, but no detectable behaviour differences from normal mice.

2 Percolated fur, huddle reflex, responds to stimuli (such as tap on cage), just as active during handing as healthy mouse.

3 Slower response to tap on cage, passive or docile when handled, but still curious when alone in a new setting.

4 Lack of curiosity, little or no response to stimuli, quite immobile.

5 Laboured breathing, inability or slow to self-right after being rolled onto back (moribund, sacrificed).

WBC and Platelets Counts: 100 µl of blood was obtained from 2 randomly selected mice per group utilising a tail bleed method at the 24 hour time-point from TSST-1 model. Whole blood was collected in EDTA tubes and analysed in an automated blood haematology analyser.

Results

Gel permeation of IR-U and IR-P: Figure 15 represents a FPLC chroatogram of 50 µl of undiluted IR-U sample. The running buffer was PBS. The chromatogram indicates 4 major peaks at 70, 37, 15 and 10 kDa. To identify these peaks, a sample of 500 µl (containing 5000 IU) of IR-P (Pregynl) was applied on the same column under similar running conditions. The profile obtained (figure 16) displayed also these 4 peaks although the ratios were different. Peak fraction 2 represents (alpha/beta) heterodimer hCG (37 kDa) while fraction 3 represents individual chains, homodimers of these chains or beta-core residual chains and other molecules (15-30 kDa). From these results we concluded that first trimester urine contains the same 4 major protein fractions that are also present in commercial hCG preparation, as could

be expected. We named them as (IR-P1, IR-P2, IR3-5[pooled]), (IR-U1, IR-U2, IR-U3-5[pooled]). Fraction 5 contains no protein or protein less than 10 kDa weight. In addition overlapping fractions 2 and 3 were seen in IR-P as well as in IR-U which suggested covalent binding of protein species present in these fractions.

Anion exchange chromatography and further treatment of IR-U and IR-P:

Further separation of the overlapping fractions 2 and 3, was done on a 1 ml MONO Q HR 5/5 anion exchange column. Figure 17 represents a chromatogram of 50 µl of IR-U sample diluted 1:20 in PBS. Two major protein peaks eluted at 43% and 55% buffer B but were not separated suggesting covalent binding between these protein species. Even using a discontinuous elution gradient with a 50% buffer B hold did not result in separation of these peaks (data not shown). Therefore, we concluded that ion exchange chromatography could not be used for further purification due to covalent binding of protein species present in the urine sample.

To reduce the presumed covalent binding between the important protein species present in the IR-U sample, we treated the sample with 60 mM 2-mercaptoethanol for 3 min at 100 ^{0}C and sample was then applied to the Superdex 75 column under identical conditions. Figure 18 represents the elution profile showing that peak 1 (70 kDa) remains present (see also figure 15-17), fraction 2 (representing hCG, 37kDa) did nearly disappear and resulted in two new peaks a low molecular weight (<10 kDa). Peak 3 remained present and therefore is likely to contain isolated beta-core and monomeric proteins is excess. Peak 4 (10 kDa) also disappeared due to the reducing treatment.

A similar reducing treatment was applied to sample of IR-P (Pregnyl). Like the profile of the IR-U sample also treated, hCG (Figure 19) displayed the decrease in

peak 2, increase in peak 3, while a new protein peak appeared between peaks 1 and 2. Moreover, an increase in the breakdown product peak (<10 kDa) was apparent.

In vivo anti-diabetic effect of IR: Four 15-wk-old NOD female mice (n=4) were treated with PBS, 300 IU Pregnyl, or 600 IU Pregnyl intraperitonealy, 3 times a week for four weeks. After the treatment all mice in the PBS group were diabetic (blood glucose >33 mmol/l), they lost weight and looked uncomfortable, while the 300 IU Pregnyl and 600 IU Pregnyl groups remained free of disease. Their blood glucose levels never exceeded 6 mmol/l and they looked very healthy (Figure 1 and 3). In order to assess possible infiltrations and intact insulin producing cells in the pancreas, mice from the PBS and the 600 IU Pregnyl groups were killed after treatment and entire pancreata were removed for immunohistochemistry for insulin. Pancreas sections from the PBS group showed many infiltrating cells in the pancreas and these cells penetrated the islets. There were also large number of B lymphocytes and T lymphocytes present in the pancreata of the PBS-group. This finding was consistent with our other finding of an elevated ratio of splenic CD8/CD4 cells due to a selective reduction in the number of CD4+ cells and a decrease in the number of B lymphocytes in the spleen of these mice (data not shown). In the 600 IU Pregnyl group, pancreata were free of infiltration and, surprisingly, a number of new insulin producing islets were seen. There was also a decrease in the number of B lymphocytes and T lymphocytes in pancreas, which was consistent with normal levels of the CD8/CD4 ratio and the number of B lymphocytes in the spleens of these mice. Mice from the 300 IU Pregnyl group were kept alive till the age of 28 weeks. They appeared healthy, did not loose their weight and never had blood glucose levels above 8 mmol/l (Figures 1 and 3). Immunohistochemistry for the

presence of insulin was also performed. There were still infiltrating cells present and some insulin producing islets in the pancreas. These mice were treated for four weeks with Pregnyl along with the 600 IU Pregnyl group and from wk 20 till 28 they were left untreated.

In order to determine whether the spleen cells of treated and untreated NOD mice still had the potential to induce diabetes in NOD.scid, we transferred spleen cells from the PBS and the 600 IU Pregnyl group into NOD.scid mice. 22 days after transferring, the PBS NOD.scid group were positive for diabetes and within a week they reached a blood glucose level above 33 mmol/l, while NOD.scid mice receiving spleen cells from the 600 IU Pregnyl group remained normal (blood glucose <7 mmol/l). 7 weeks after transferring, the PBS group looked very uncomfortable (Figure 2.), while the 600 IU Pregnyl. NOD.scid group still had blood glucose levels less than 9 mmol/l and remained healthy. Mice from both groups were killed at this time.

In vitro restimulation. Since high levels of IFN-γ, IL-1, and TNF-α were reported during the course of disease in NOD and this cytokine profile fits in a selective activation of the Th1 subset, we tested in vitro the effect of Pregnyl on cytokine production by total spleen cells and purified CD4+ cells from 20-wk-old NOD female mice. In order to assess whether the anti-diabetic effect resides in hCG or in one of its subunits or in other factors contained in the preparation used , we also tested the effect of different fractions obtained by gelpermeation chromatography from Pregnyl (Figure 12) and human recombinant hCG and its subunits on cytokine production. The effect of these fractions were also tested in vivo on blood glucose levels in reconstituted NOD.scid mice.

We observed a strong inhibition of IFN-γ production by spleen cells obtained from mice treated with 50-600 IU/ml of Pregnyl, F3-5 (58-15 Kda) and to a lesser extent with human recombinant-βCG (Figures 4-6). There was only a moderate increase in IFN-γ production splenocytes from mice treated with 800 IU/ml Pregnyl. A similar pattern was observed when analyzing IL-4 production (Figure 5). In addition a marked inhibition of IL-1 and TNF-α production was observed in stimulated splenocytes from mice treated with 300-600 IU/ml Pregnyl, with a concomitant stimulation of IL-6 and IL-10 production (data not shown).

Furthermore, transfer experiments showed that total spleen cells of 20-wk-old NOD mice treated with F3-5 or 600 IU Pregnyl can delay or even prevent the onset of diabetes in NOD.scid as compared to reconstitution with PBS treated NOD cells (Figure 7). However, no significant effect was observed with F1-2 (80-70 Kda) on the onset of diabetes in NOD.scid mice. In order to test whether Pregnyl has also effect on Th2 type mice, we treated BALB/c mice (n=5) with 300 IU Pregnyl i.p. for four days and with PBS (n=5). After isolating CD4+ cells from spleens we stimulated them with anti-CD3/IL-2 for 48 hours and the supernatants were collected for the determination of IFN-γ and IL-4 cytokines. We also treated CD4+ cells with different doses of Pregnyl. Subsequently the supernatants were collected for cytokine anaylses. There was a marked inhibition of IFN-γ and a concomitant stimulation of IL-4 found in CD4+ cells stimulated with anti-CD3/IL-2 only (Th1->Th2), while the inverse was seen in CD4+ cells treated in vitro with different doses of Pregnyl (Th2->Th1).

In-vivo anti-sepsis or septic shock effect of IR

Survival Curve: The most striking results from this experiment are the black and white difference between those animals treated with IR-P prior to TSST-1 and D-Gal treatment versus those that were not (Figure 20.). This is evident in the survival curve obtained from this experiment. While a 4 µg dose of TSST-1 coupled with D-Galactosamine sensitisation was 100% lethal by 32 hours; animals pretreated with IR prior to TSST-1 exposure did not succumb to the effects of lethal toxic shock.

LPS treated Balb/c mice and SJL mice revealed different sensitivity to LPS. 600 µg LPS was 100% lethal by 48 hours and 36 hours in Balbc and SJL, respectively, while IR pre-treated Balb/c and SJL mice remained alive. We also pre-treated Balb/c mice with IR-U fractions namely, IR-U1, IR-U2 and IR-U3-5[pooled] and then treated with LPS. These experiments showed that IR-U1 and IR-U2 pre-treated mice were very sick by 48 hours and were killed along with LPS group. However, mice treated with IR-U3-5 remained alive.

A group of Balb/c mice were treated twice with 700 IU IR-P after the injection of LPS. The control group mice (only LPS) were killed at 48 hours time point because of their severe sickness. Mice treated with IR-P remained alive, except two (2/6) mice were killed at 60 hours time point.

Illness Kinetics: Visible signs of sickness were apparent in all of the experimental animals, but the kinetics and obviously the severity of this sickness were significantly different: like IR-P pretreated Balb/c mice group did not exceed the sickness level 2 in TSST-1 exotoxin model (Figure 21.) and also in LPS endotoxin model in addition to IR-U3-5 pre-treated mice. IR-P pre-treated SJL mice and IR-P post-treated Balb/c mice in LPS model did not exceed the sickness level 3. All mice in

both models were killed when they exceed the sickness level 5.

Shock Induced Weight Loss in TSST-1: IR pretreatment also resulted in significantly reduced weight loss of survivors of toxic shock. Weight loss data from this experiment was combined with that from another experiment which followed identical illness kinetics (data not shown), but resulted in two survivors of the 4ug TSST-1 &D-Gal without IR pre-treatment group. (Figure 12.).

When this weightloss data was statistically analysed using a 2-sample T-test (using Minitab statistical software, version 11.21) significant differences ($P(HO:\mu1=\mu2)<0.05$) in weight loss were observable at 32 and 48 hours despite low n numbers, indicating an even higher possible significance if n were increased:

Two Sample T-Test and Confidence Interval

Two sample T for weight loss at 32 hours
(group 1=TSST1&D-Gal;group 2=T&D with IR pre-treatment)

group	Mean	StDev	SE Mean	
1	4	4.75	1.79	0.89
2	6	1.28	2.22	0.91

95% CI for $\mu1 - \mu2$: (0.45, 6.48)
T-Test $\mu1 = \mu2$ (vs not =): T= 2.72 **P=0.030** DF= 7

Two sample T for weight loss at 48 hrs
(group 1=TSST1&D-Gal;group 2=T&D with IR pretreatment)

group	N	Mean	StDev	SE Mean	
1	3	10.05	2.25	1.3	
2	6	3.49	4.41	1.8	

95% CI for μ1 - μ2: (1.1, 12.0)

T-Test μ1 = μ2 (vs not =): T= 2.95 **P=0.026** DF= 6

WBC and Platelets Counts: White blood cell levels in blood (Figure 23) were significantly higher in TSST- and D-Gal alone treatment (bar #2) versus WBC counts in regular mice (bar #1) and IR-P pre-treated mice (bar #3). This indicates, as expected, a higher level of immune activation in the mice suffering from lethal toxic shock. There is still a normal level of WBC in the IR-P group, such a finding also fits our other results, as this group did not show severe visible signs of illness.

Blood platelet counts (Figure 24) were also reduced in TSST-1 D-Gal treated mice. Elevated platelet counts were seen in IR-P treated mice.

Discussion

Nonobese diabetic (NOD) mice naturally develop an insulin-dependent diabetes (IDDM) with remarkable similarity in immunopathology and clinical symptoms to human IDDM patients. As a result, NOD mice have become a valuable tool for studying the underlying immunobiology of IDDM and the complex genetics that control it. Through their study we now know that diabetes is caused by a disbalance in the ratio of the Th1/Th2 subsets and consequently, the destruction of insulin producing β-cells. This destruction is co-ordinated by β-cell antigen-specific CD4+ T cells that produce proinflammatory cytokines like IFN-γ, TNF-α/β, and IL-1. A growing number of studies has now correlated diabetes (in

mice and in humans) with a preferential development of Th1-like cells.

In contrast, pregnancy is thought to be a selective Th2 phenomenon, and surprisingly during pregnancy the severity of many immune-mediated diseases has been seen reducing. In contrast, Gallo et al. have shown that hCG mediated factor(s) (HAF) present in the urine of first trimister pregnancy have an anti-tumour (and anti-viral) effect, which is possibly achieved by a direct cytotoxic effect on tumour cells and, according to these authors, not by an immune-mediated response.

Here we show an immunoregulator obtainable for example from urine of (first trimester) pregnancy not only effects the above mentioned immune deviation during pregnancy, but also effects the development of diabetes in NOD mice.

Our results show that for example Pregnyl, a partially purified hCG preparation from urine of first trimester pregnancy, can delay the onset of diabetes, for example in 15-wk-old NOD when treated only for 3 times a week during four weeks. In addition, spleen cells isolated from these treated mice upon transfer have also the potential to delay the onset of diabetes in immuno-compromised NOD.scid mice. We fractionated a Pregnyl preparation to assess whether this anti-diabetic activity resides in hCG itself, its subunits, β-core (naturally break-down product of β-hCG) or in unidentified factors (HAF). It is worth knowing that Pregnyl is one of the most purified hCG preparations available and it contains only low amounts of β-core fragments. We found that most of the anti-diabetic activity resided in a fraction without hCG. Furthermore, we showed that human recombinant α-hCG and β-hCG also had no effect. However, we do not exclude the possibility that hCG can synergize

with other factors in diabetes and other immune mediated diseases.

Immunohistological analysis of the presence of insulin and infiltration in the pancreas of NOD mice showed that NOD mice treated with 600 IU Pregnyl did not reveal a significant infiltrate. Moreover, new insulin islets were seen the in pancreas, which shows a possible regeneration process induced by this treatment. As mentioned before, normally at the age of 9 weeks infiltrating cells penetrate into the islets and the islets become swollen with lymphocytes. In our experiments, the NOD mice were 15-wk-old and the PBS treated control mice had many infiltrating cells and almost no insulin producing cells at that time in their pancreas. In addition, PBS treated mice had also an elevated ratio of CD8/CD4 in their spleen and many T cells in their pancreas. Since our treated mice had a normal CD8/CD4 ratio in their spleen and no infiltration was found in their pancreas, the elevated CD8/CD4 ratio was due to selective recruitment of CD4+ cells into the pancreas. IFN-γ and TNF-α are involved in the recruitment of T lymphocytes (Rosenberg et al. 1998).

Our results show that treatment of NOD mice with 600 IU Pregnyl for four weeks had dramatic effects on the morphology and function of their otherwise inflamed pancreas. Furthermore, our 300 IU Pregnyl NOD mice were kept alive till the age of 28 weeks without treatment and remained non-diabetic. The 600 IU Pregnyl NOD mice were also examined for symptoms of generalised auto-immune diseases, like Sjögren's disease, which were not found.

Our in vitro experiments with total spleen cells and purified CD4+ cells of NOD are consistent with the in vivo data. There was marked inhibition of IFN-γ, IL-1 and TNF-α release by spleen cells (data not shown) from NOD mice treated in vitro with Pregnyl, F3-5, and to lesser extent with human recombinant β-hCG. Increase in IL-4

production was also observed implying a shift of Th1 to Th2 type response with the treatment. However, doses above 800 IU Pregnyl caused opposite results and may be due to the presence of high amount of hCG itself.

The immune system is clearly involved in the onset of diabetes. Treatment with Pregnyl effects the immune system and thereby can reduce the disease activity in NOD mice. In order to separate the immune-modulating activity of Pregnyl from its benefical clinical effect, we treated healthy BALB/c mice. This strain is generally considered to react upon stimulation with a Th2 driven immune response. Our results suggest that purified CD4+ T cells obtained from Pregnyl-treated BALB/c mice display a further Th2 skewing. The same cells when restimulated with Pregnyl in vitro showed an enhancement of IFN-γ production and a decrease in IL-4 production. This implies that Pregnyl effects different regulatory T cells subsets upon treatment in vivo versus in vitro. We suggest that treatment in vivo stimulates the outgrowth of a population of presumably CD4+ Tr1 cells, characterised by selective production of TGF-β and a lower or no production of IL-10. These CD4+ Tr1 cells have been shown (O'Garra et al. 1997) in different models of Th1 driven diseases including diabetes and MS, to selectively inhibit the activity of Th1 cells, thereby decreasing the disease severity also. Similar by CD4+ T cells from Pregnyl treated BALB/c mice restimulated in vitro with Pregnyl showed an increase of Th1 cells concomitant with a decrease of Th2 cells. This is consistent with a preferential stimulation of the CD4+ Th3 cells characterized by a high production of IL-10 and a low production of TGF-β. These regulatory cells are inhibitors of IFN-γ production by Th1 cells as well as the outgrowth of Th2 type cells. It has been also shown that in NOD.scid mice a steady increase of Th2 cells is

responsible for the less severe hyperglycemia and the different nature of the infiltrates in the pancreatic islets.

Our results of the 300 IU Pregnyl treated NOD and our reconsituted NOD.scid mice showed a similar slow increase in blood glucose, particularly in NOD.scid, and a different nature of the infiltrates as compared to PBS-treated NOD. In NOD mice the activity of Pregnyl might well be mediated with the induction of Th3 cells inhibiting both Th1 and Th2 cells. These Th3 cells may suppress the disease activity for prolonged periods of time at the very least. In NOD.scid mice, having no functional T cells, reconstitution with Pregnyl-treated spleen cells is mediated with selective induction of Tr1 cells and thereby inhibiting the Th1 subset only. After prolonged periods the steady outgrowth of diabetogenic Th2 cells is responsible for the late onset of a less severe form of diabetes. Similarly our F3-5, but not F1-2, displays the above discussed phenomenon, arguing that hCG can not be responsible for the observed effects. This F3-5 is principally pointing towards a decisive effect on the immune response in the onset of auto-immune diabetes and is an active component for immunotherapy of this disease and other immune mediated disorders.

In addition, Pregnyl and immunoregulators functionally equivalent thereto, is effective in Non-insulin-diabetes mellitus (NIDDM). The essential problem in NIDDM patients is the insulin resistancy and obesity, it has been shown that TNF-(alpha) is the cause of the insulin resistance of obesity and NIDDM (Miles et al. 1997, Solomon et al. 1997, Pfeiffer et al. 1997, Hotamisligil et al. 1994), Argiles et al. 1994). This insulin resistance induced by TNF-alpha can be reversed by recently developed medicines like Pioglitazone and Metformin, and with engineered human anti-TNF-alpha antibody (CDP571) (Solomon et al. 1997, Ofei et al.

1996), which possibly achieved their benefical action by lowering TNF-alpha induced free fatty acids (FFA) concentration of the blood and/or by stimulating glucose uptake at an intracellular point distal to insulin receptor autophosphorylation in muscle. Furthermore, the presence of retinopathy (Pfeiffer et al. 1997) (one of the late complications of diabetes) has been mediated with significantly elevated plasma TNF-alpha and is sex-dependent (Pfeiffer et al. 1997). The increased TNF-alpha occurs in male but not in female NIDDM and may participate in the development of retinopathy and other complications like neuropathy, nephropathy or macroangiopathy (Pfeiffer et al. 1997). Since Pregnyl and fraction 3-5 have immune modulating potential and in particular inhibit TNF-alpha directly or indirectly, Pregnyl and its fraction 3-5 have also benefical effects in NIDDM patients. Besides, lower incidence of diabetes complications among female could implicate the involvement of female hormones. A key pathogenic cytokine indicated in sepsis or septic shock is the immunological mediator TNFα which occupies a key role in the patho-physiology associated with diverse inflammatory states and other serious illnesses including sepsis or septic shock and cachexia. When TNF is produced by T cells (for example by T cell activation through superantigen [exotoxin]) or by macrophages through endotoxin), it mediates an inflammatory response that may alienate and repel the attacking organisms. When the infection spreads, the subsequent release of large quantities of TNF into the circulation is catastrophic, damaging the organ system and triggering a state of lethal shock. These toxic effect occur by direct action of TNF on host cells and by the interaction with cascade of other endogenous immunological mediators including IL-1, IFN-gamma.

This has been shown by induction of shock like symptoms in mice sensitised with D-Galactosamine and treated with TNFα as well as inhibition of both lethality and visible signs of disease after concurrent infusion of anti-TNFα mAbs following TSST-1 and D-Galactosamine treatment. In the low dose endotoxin model and in exotoxin model, D-Galactosamine treatment is necessary to inhibit the transcription of acute phase proteins that allow the liver to detoxify the high levels of TNFα present following shock induction. The lack of these acute phase proteins leads to increased susceptibility of murine hepatocytes to TNFα mediated apoptosis induction. This apoptosis, and inability to neutralise the inflammatory effects of TNFα eventually lead to death.

We have shown that factors (IR) with or without hCG present in for example the urine of first trimester of pregnancy (IR-U) and in commercial hCG preparations (IR-P) have immune regulatory effects. In particular, they have the potential to inhibit auto-immune and inflammatory diseases. Since TNF and IFN-gamma are pathologically involved in sepsis or septic shock and also in auto-immune and inflammatory diseases, IR has also the ability to inhibit TNF and IFN-gamma in acute inflammatory states like shock. Our results show that IR inhibits sepsis or septic shock in BALB/c or SJL, treated with LPS (endotoxin model) or with TSST-1 (exotoxin model). IR has not only the potency to inhibit chronic inflammatory diseases but it can also suppress acute inflammatory diseases like shock. Moreover, we also show that even post-treatment with IR inhibits the shock. Furthermore, our IR fraction data show that most of the anti-shock activity resides in fractions IR-(U/P)3-5[pooled] which contain mostly individual chains of hCG, homodimers of these chains or beta-core residual chains, breakdown products of these chains and other molecules

(>30 kDa). We have also shown that the same fractions IR-U/P3-5 have anti-diabetic effect in NOD mice model. Thus the endotoxin and exotoxin model serves as a fast readout model for the determination of anti-diabetic activity in NOD mice and NOD.scid mice. With the help of endotoxin and exotoxin model we can check for anti-diabetic activity in IR fractions within 48 hours.

Thus, IR such as Pregnyl and its fraction 3-5 have high potency to suppress auto-immune diabetes by modulating the immune system by effecting regulatory T cells subsets. Our NOD and BALB/c data show that they have the potential to restore the T-cell subset balance (Th1->Th2/Th2->Th1). Therefore, Pregnyl and its fraction 3-5 are effective in modulating the severity of other immune-mediated diseases too, like diseases where Th1 cytokines are dominant such as Rheumatoid Arthritis (RA), Multiple Sclerosis (MS), NIDDM, Systemic lupus erythematosus (SLE), transplantation models and diseases like allergies and asthma where Th2 cytokines responses are dominant. Animal models of these diseases (like EAE-model for MS, BB-rats for NIDDM, Fishe-rat and MLR-models for RA, OVA-model for allergies, MLR/lpr and BXSB-models for SLE), KK-Ay-mice, GK rats, wistar fatty rats, and fa/fa rats provide, amongst others, models of other immune-mediated diseases.

Figures

Figure 1. Shows that 15-weeks-old NOD mice treated with PBS for 4 weeks, become diabetic (>13.75 mmol/l) at the age of 17 weeks and within a week they had blood glucose levels above 30 mmol/l, while NOD mice treated with 300 Pregnyl remained nondiabetic till they were killed (at the age of 28-weeks) even the treatment was stopped at age 19 weeks. There blood glucose level remained lower than 8 mmol/l.

Figure 2. shows that reconsituted NOD.scid mice receiving spleen cells from PBS treated NOD mice(fig.3) became diabetic after 22 days of transferring, while reconsituted NOD.scid mice with 600 IU Pregnyl treated NOD remained nondiabetic till they were killed (8 weeks after transferring).

Figure 3. Shows that 15-weeks-old NOD mice treated with PBS for 4 weeks, become diabetic (>13.75 mmol/l) at the age of 17 weeks and within a week they had blood glucose levels above 30 mmol/l, while NOD mice treated with 600 IU Pregnyl remained nondiabetic till they were killed along with PBS group (at the age of 21-weeks). 15-weeks-old NOD mice treated with 300 IU Pregnyl remained nondiabetic till they were killed (at the age of 28-weeks) even the treatment was stopped at age 19 weeks. There blood glucose levels remained lower than 8 mmol/l.

Figure 4. Spleens cells from 20-weeks-old female NOD were islolated and were cultured for 48hrs with different conditions ('-' only medium, '+' with anti-CD3,50, 100, 300, 600, 800 IU/ml Pregnyl, F1-2, F3-5, rh-hCG, rh-alpha-hCG, rh-beta-hCG [each at 200ug/ml]) in the presence of anti-CD3 and IL-2. After 48hrs INF-(cytokine ELISA were done. Results shows that there is

dose dependent inhibition of INF-(with Pregnyl (50-600 IU/ml) and fraction 3-5 (F3-5) containing no hCG. There is an increase in INF-g with 800 IU/ml Pregnyl which suggests the effect of hCG itself. NO effect on INF-g were seen with fraction 1-2 (F1-2) containing hCG, human recombinant(rh) hCG, rh-alpha-hCG. Slight decrease in INF-g level is seen with rh-beta-hCG.

Figure 5. Spleens cells from 20-weeks-old female NOD were isolated and were cultured for 48hrs with different conditions ('-' only medium, '+' with anti-CD3,50, 100, 300, 600, 800 IU/ml Pregnyl, F1-2, F3-5, rh-hCG, rh-alpha-hCG, rh-beta-hCG [each at 200µg/ml]) in the presence of anti-CD3 and IL-2. After 48hrs IL-4 cytokine ELISA was done. Results shows that there is a dose dependent increase of IL-4 with Pregnyl (50-600 IU/ml) and fraction 3-5 (F3-5) containing no hCG. There is an decrease in IL-4 with 800 IU/ml Pregnyl which suggests the effect of hCG itself. NO effect on IL-4 were seen with fraction 1-2 (F1-2) containing hCG, human recombinant(rh) hCG, rh-alpha-hCG and rh-beta-hCG.

Figure 6. CD4 T-cells from spleen of 20-weeks-old female NOD were islolated and were cultured for 48hrs with different conditions ('-' only medium, '+' with anti-CD3,50, 100, 300, 600, 800 IU/ml Pregnyl, F1-2, F3-5, rh-hCG, rh-alpha-hCG, rh-beta-hCG [each at 200µg/ml]) in the presence of anti-CD3, IL-2 and anti-CD28. After 48hrs INF-γ cytokine ELISA were done. Results shows that there is dose dependent inhibition of INF-γ with Pregnyl (50-300 IU/ml) and fraction 3-5 (F3-5) containing no hCG. There is an increase in INF-γ with 600- 800 IU/ml Pregnyl which suggests the effect of hCG itself. NO effect on INF-g were seen with fraction 1-2 (F1-2) containing hCG, human recombinant(rh) hCG, rh-alpha-hCG. Slightly decrease in INF-γ level is seen with rh-beta-hCG.

Figure 7. Show the transfer experiment of 20-weeks old female spleen cells treated with PBS, 600 IU Pregnyl, fraction 1-2(F1-2), Fraction 3-5(F3-5) or human recombinant beta-hCG (b-hCG) for 48hrs and then transferred into 8-weeks old NOD.scid (n=3). After 22 days of transfer the NOD.scid mice receiving PBS treated NOD spleens were diabetic. NOD.scid mice receiving F1-2 and b-hCG were diabetic after 4 and 5 weeks respectively while NOD.scid mice receiving 600 IU Pregnyl and F3-5 remained nondiabetic about 6 weeks and then all mice were killed. It shows that the maximum antidiabetic effect resides in Pregnyl and F3-5. Since F1-2 which contain mostly hCG have no effect on the incidence of diabetes in these mice, it is clear that antidiabetic effect does not reside in hCG itself. There is slightly anti-diabetic affect in recombinant human beta-hCG.

Figures 8-11

In order to test whether Pregnyl has also effect on Th2 type mice, we treated BALB/c mice (n=5) with 300 IU Pregnyl i.p. for four days and with PBS (n=5). After isolating $CD4^+$ cells from spleens we stimulated them with anti-CD3/IL-2 for 48 hours and the supernatants were collected for the determination of IFN-γ (figure 8) and IL-4 (figure 9) cytokines. We also treated $CD4^+$ cells with different doses of Pregnyl. Subsequently the supernatants were collected for INF-g ELISA (Figure 10) analyses. Figure 8 shows the invivo treatment with 300 IU Pregnyl suppress INF-g and on the other hand increases IL-4 production. This implies that there is more shift towards Th-2 phenotype. Same cells treated again in vitro with different dosis of Pregnyl show (Figure 10) increase in INF-g and decrease in IL-4 (figure 11) which suggest the shift towards Th-1 phenotype. This all implies that

Pregnyl and F3-5 have affect on regulatory T-cell subset (Th3, Tr1).

Figure 12

column

Superdex 75 HR 10/30; FPLC system (Pharamcia)
total volume V_t= 25 ml; void volume V_0=8.7ml; flow rate: 1 ml/min; buffer: 10mM
phosphate-buffered saline, pH 7.3; at room temperature
column efficiency=38,000 N/m
selectivity K_{AV} = 1.737 - 0.2782 log (r^2 = 0.982), MW = molecular mass
separation range: 3,000 - 100,000 Dalton for globular proteins

running method

```
METHOD NO. 4
  0.0 CONC%B      0.0
  0.0 ML/MIN      0.20
  0.0 CM/ML       0.20
  0.5 ML/MIN      0.50
  0.5 CM/ML       0.50
  0.8 ML/MIN      1.00
  2.0 CLEAR DATA
  2.0 ALARM       0.1
  2.0 HOLD
  2.0 VALVE.POS   1.2
  2.0 MONITOR       1
  2.0 LEVEL %     5.0
  2.0 ML/MARK     2.0
  2.0 INTEGRATE     1
  4.0 VALVE.POS   1.1
  6.0 PORT.SET    6.0
 30.0 INTEGRATE     0
 45.0 CONC %B     0.0
```

sample

Pregnyl (Organon, lot nr.:168558, exp.date:28.11.99) sample volume = 0.5 ml = 2,000 units; sensitivity 0.1 AUFS

chromatogram

Peak 1 = fractions 1-2: Ve = 14.7 - 15.1 ml; K_{AV}=0.37-0.39

Peak 2 = fractions 3-5: Ve = 15.38 - 17.99 ml;

K_{AV} = 0.41 - 0.57

$K_{AV} = (V_e - V_0) / (V_t - V_0)$

Peak 1 elutes at a volume between 14.7 - 15.1 ml after start of the separation. This corresponds to a molecular mass between 70,000 - 80,000 Dalton. This fraction contains in part the dimeric form of hCG (Textbook of Endocrine Physiology, Second edition, J.E. Griffin, S.R. Ojeda (Ed.) Oxford University Press, Oxford, 1992, pp.199). Peak 2 elutes at a volume between 15.38 and 17.99 ml, corresponding to a volume between 1500 - 58,000 Dalton. This fraction contains partly β-subunit (MW=22,200 Dalton), breakdown products of hCG and other, as yet, unknown molecules. These calculations were based on the above-mentioned selectivity of this column.

Figure 13. Prosposed mechanisms operating in three different models of sepsis or septic shock. A) is a high-dose endotoxin model. B) is a low-dose endotoxin model. C) is exotoxin model for TSST-1/SEB. In high and low-dose endotoxin model (a,b) the systemic effects of endotoxin (LPS) is largely mediated by macrophages while in exotoxin model (c) the systemic effects of supper antigen (TSST-1/SEB) is mediated by T-cells. In both cases production of TNF, IFN and ICE (IL-1 alpha and beta) play important role in the pathogenesis of septic shock.

Figure 14. T-cell activation induced by super-antigens like TSST-1 can be seen as a polyclonal T-cell activation in that T-cells expressing a specific V-beta family are all activated through non antigen specific binding of the TCR/MHCII/ and superantigen.

Figure 15. An FPLC chromatogram of 50 µl of undiluted IR-U sample.

Figure 16. An FPLC chromatogram of 500 µl of undiluted IR-P sample.

Figure 17. Further separation of fractions 2 and 3 from figure 15.

Figure 18. An FPLC chromatogram of 50 µl 2-mercapto ethanol treated IR-U sample.

Figure 19. An FPLC chromatogram of 500 µl 2-mercapto ethanol treated IR-P sample.

Figure 20. A black and white difference in survival between those animals treated with IR-P prior to TSST-1 and D-Gal treatment versus those that were not is found.

Figure 21. IR-P pretreated Balb/c mice group did not exceed the sickness level 2 in TSST-1 exotoxin model while D-Gal-TSST-1 group exceed the sickness level 5 and were killed.

Figure 22. IR pretreatment also resulted in significantly reduced weight loss of survivors of toxic shock.

Figure 23. This figure indicates a higher level of immune activation in the mice suffering from lethal toxic

shock (bar#2). There is still a normal level of WBC in the IR-P group (bar#3) as compared to normal Balb/c mice (bar#1).

Figure 24. This figure indicates slight reduction in platelets count in TSST-1 group (bar#2) as compared to normal Balb/c mice (bar#1). The platelets count were seen very high in IR-P treated group Balb/c mice (bar#3).

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Claims

1. An immunoregulator comprising an active component obtainable from a mammalian chorionic gonadotropin preparation present in a fraction which elutes with an apparent molecular weight of 58 to 15 kilodalton as determined in gel-permeation chromatography, said active component capable of stimulating splenocytes obtained from a non-obese diabetes (NOD) mouse, or comprising an active component functionally related to said active compound.

2. An immunoregulator according to claim 1 wherein said stimulated splenocytes are capable of delaying the onset of diabetes in a NOD-severe-combined-immunodeficient mouse reconstituted with said splenocytes.

3. An immunoregulator according to claim 1 or 2 wherein said active component is capable of inhibiting gamma-interferon production of splenocytes obtained from a non-obese diabetes (NOD) mouse.

4. An immunoregulator according to anyone of claims 1 to 3 wherein said active component is capable of stimulating interleukine-4 production of splenocytes obtained from a non-obese diabetes (NOD) mouse.

5. Use of an immunoregulator according to anyone of claims 1-4 for the production of a pharmaceutical composition for the treatment of an immune-mediated-disorder.

6. Use according to claim 5 wherein said immunoregulator comprises a hCG preparation or a fraction derived thereof.

7. Use according to claim 5 or 6 wherein said disorder comprises diabetes.

8. Use according to claim 5 or 6 wherein said disorder comprises sepsis.

9. Use according to claim 5 or 6 wherein said disorder comprises postpartum thyroid dysfunction.

10. A pharmaceutical composition for treating an immune-mediated disorder comprising an active component having an apparent molecular weight of 58 to 15 kilodalton as obtainable by gel-permeation chromatography, said active component capable of stimulating splenocytes obtained from a non-obese diabetes (NOD) mouse, said stimulated splenocytes delaying the onset of diabetes in a NOD-severe-combined-immunodeficient mouse reconstituted with said splenocytes, or comprising an active component functionally related to said active component.

11. A pharmaceutical composition for treating an immune-mediated disorder according to claim 10 wherein said active component is capable of inhibiting gamma-interferon production or stimulating interleukine-4 production of splenocytes obtained from non-obese diabetes (NOD) mouse.

12. A pharmaceutical composition for treating an immune-mediated disorder according to claim 10 or 11 obtainable from a pregnant mammal, preferably a human.

13. A pharmaceutical composition for treating an immune-mediated disorder according to claim 12 obtainable from a clinical grade hCG preparation or a fraction derived thereof.

14. A method for treating an immune-mediated-disorder comprising subjecting an animal to treatment with at least one immunoregulator according to any one of claims 1 to 4.

15. A method according to claim 14 wherein said disorder comprises diabetes.

16. A method according to claim 14 wherein said disorder comprises sepsis.

17. A method according to claim 14 wherein said disorder comprises postpartum thyroid dysfunction.

18. A method according to any one of claims 14 to 17 further comprising regulating relative ratios and /or cytokine activity of lymphocyte subset-populations in said animal.

19. A method according to claim 18 wherein said subset-populations comprise Th1 or Th2 cells.

Abstract

The invention relates to the field of immunology, more specifically to the field of immune-mediated disorders such as allergies, auto-immune disease, transplantation-related disease or inflammatory disease. The invention provides an immunoregulator comprising an active component having an apparent molecular weight of 58 to 15 kilodalton as determined in gel-permeation chromatography, said active component capable of stimulating splenocytes obtained from a non-obese diabetes (NOD) mouse, said stimulated splenocytes delaying the onset of diabetes in a NOD-severe-combined-immunodeficient mouse reconstituted with said splenocytes, or comprising an active component functionally related thereto, for treatment of immune-mediated disease.

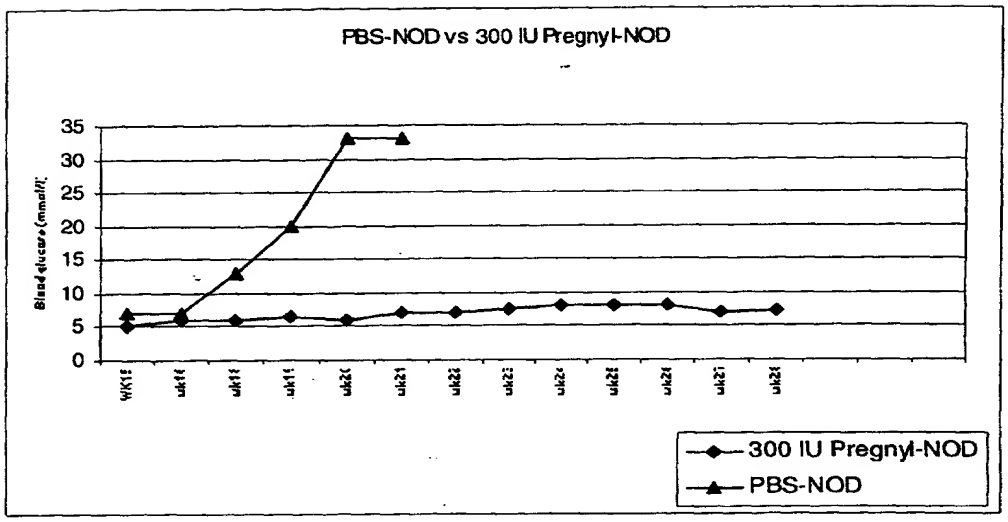


Figure 1.

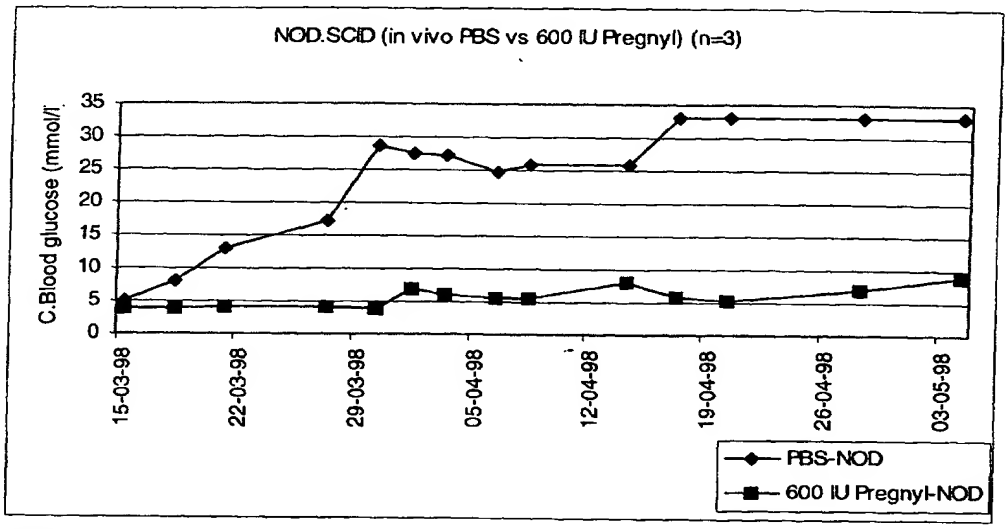

NOD.SCID (in vivo PBS vs 600 IU Pregnyl) (n=3)
C.Blood glucose (mmol/l)
35
30
25
20
15
10
5
0
15-03-98
22-03-98
29-03-98
05-04-98
12-04-98
19-04-98
26-04-98
03-05-98
PBS-NOD
600 IU Pregnyl-NOD

Figure 2.

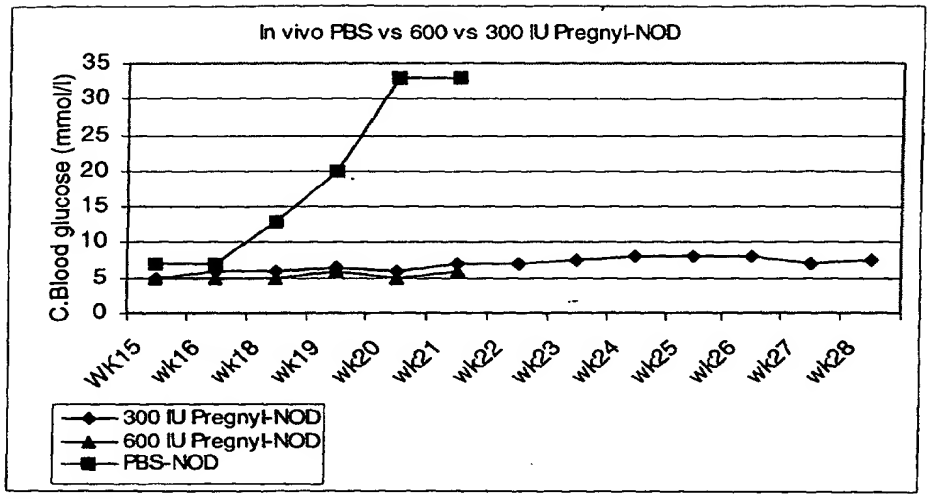

In vivo PBS vs 600 vs 300 IU Pregnyl-NOD
C.Blood glucose (mmol/l)
35
30
25
20
15
10
5
0
WK15
wk16
wk18
wk19
wk20
wk21
wk22
wk23
wk24
wk25
wk26
wk27
wk28
300 IU Pregnyl-NOD
600 IU Pregnyl-NOD
PBS-NOD

Figure 3.

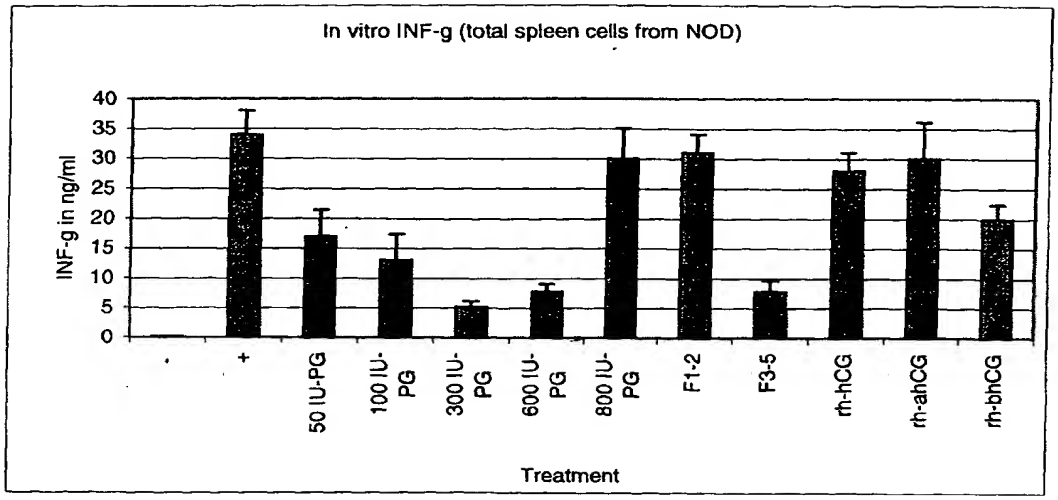

In vitro INF-g (total spleen cells from NOD)
INF-g in ng/ml
40
35
30
25
20
15
10
5
0
-
+
50 IU-PG
100 IU-PG
300 IU-PG
600 IU-PG
800 IU-PG
F1-2
F3-5
rh-hCG
rh-ahCG
rh-bhCG
Treatment

Figure 4.

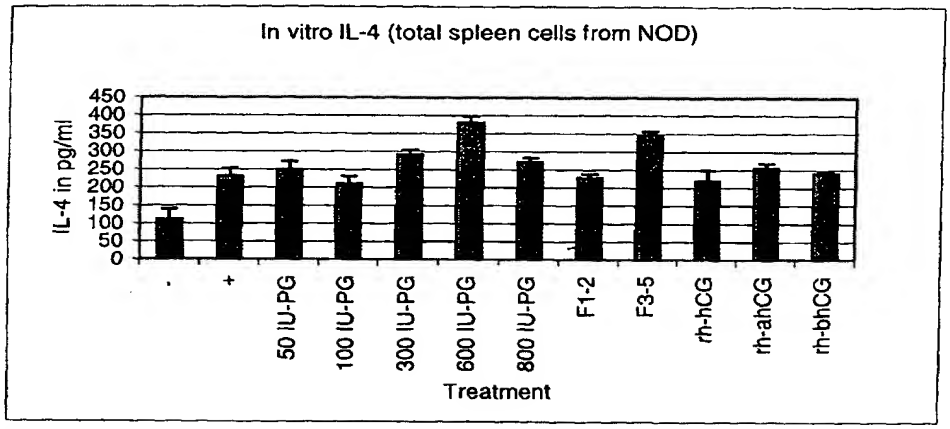

In vitro IL-4 (total spleen cells from NOD)
IL-4 in pg/ml
450
400
350
300
250
200
150
100
50
0
-
+
50 IU-PG
100 IU-PG
300 IU-PG
600 IU-PG
800 IU-PG
F1-2
F3-5
rh-hCG
rh-ahCG
rh-bhCG
Treatment

Figure 5.

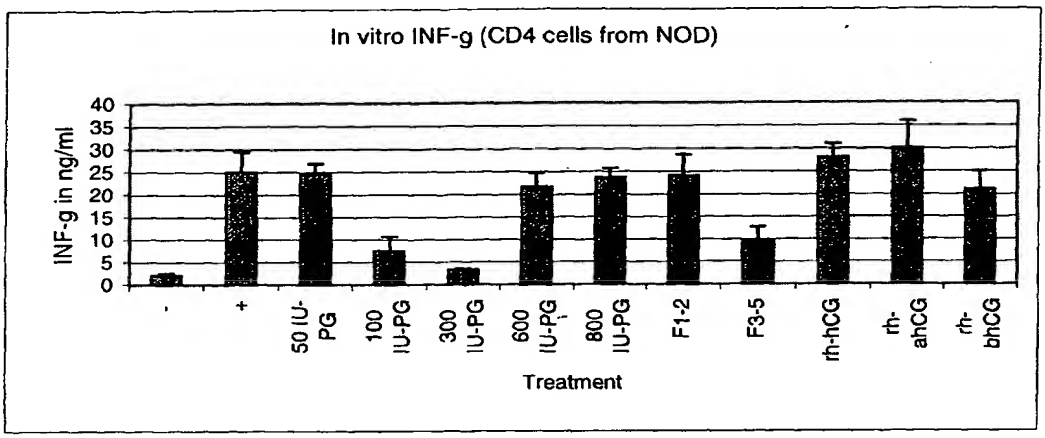

In vitro INF-g (CD4 cells from NOD)
INF-g in ng/ml
40
35
30
25
20
15
10
5
0
-
+
50 IU-PG
100 IU-PG
300 IU-PG
600 IU-PG
800 IU-PG
F1-2
F3-5
rh-hCG
rh-ahCG
rh-bhCG
Treatment

Figure 6.

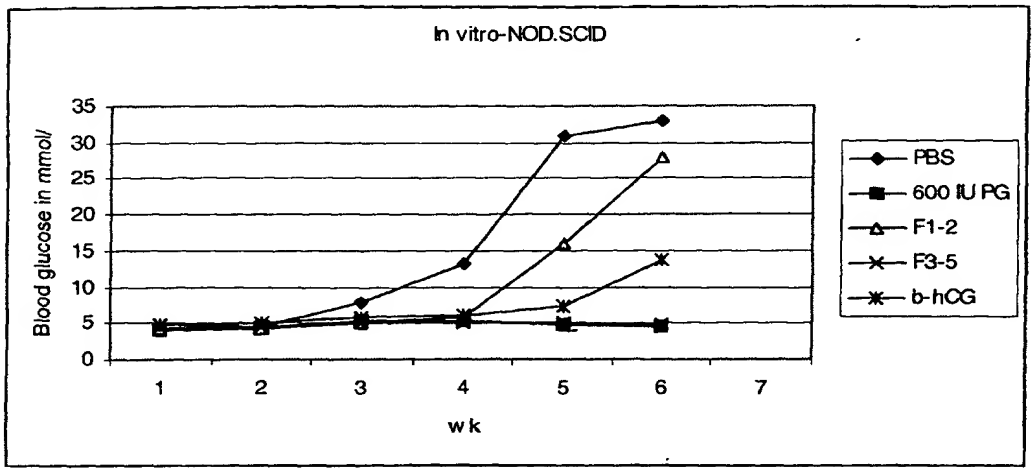

In vitro-NOD.SCID
Blood glucose in mmol/
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
wk
PBS
600 IU PG
F1-2
F3-5
b-hCG

Figure 7.

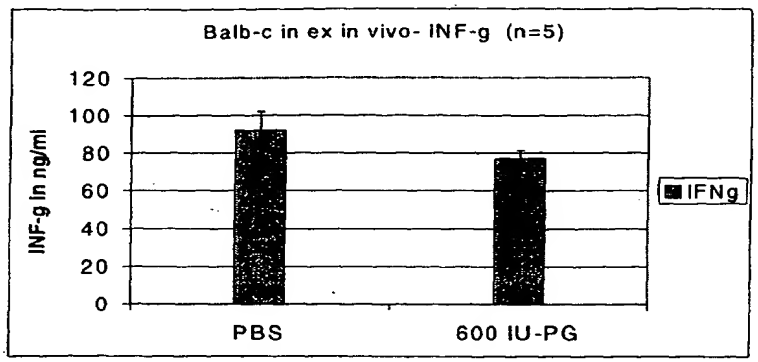


Figure 8.

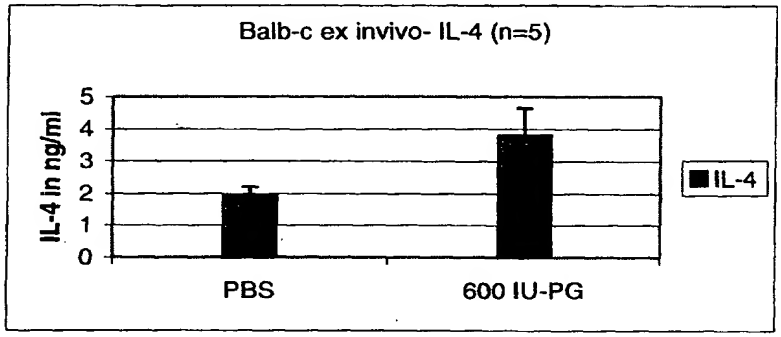


Figure 9.

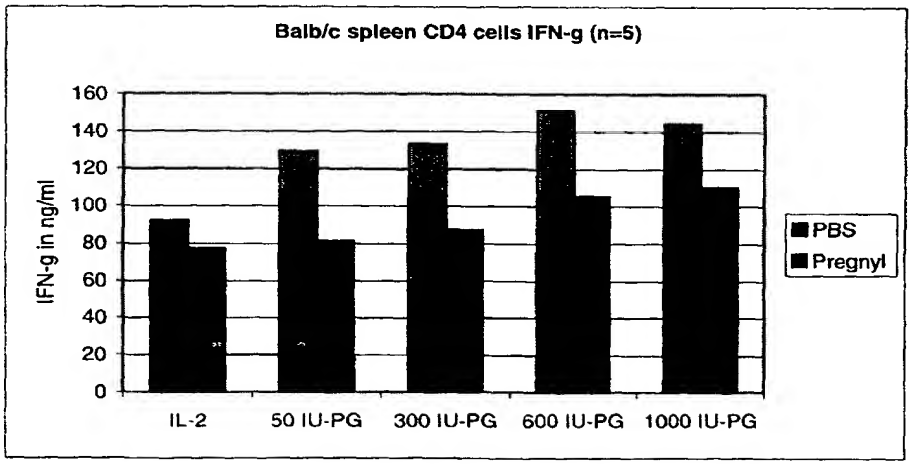


Figure 10.

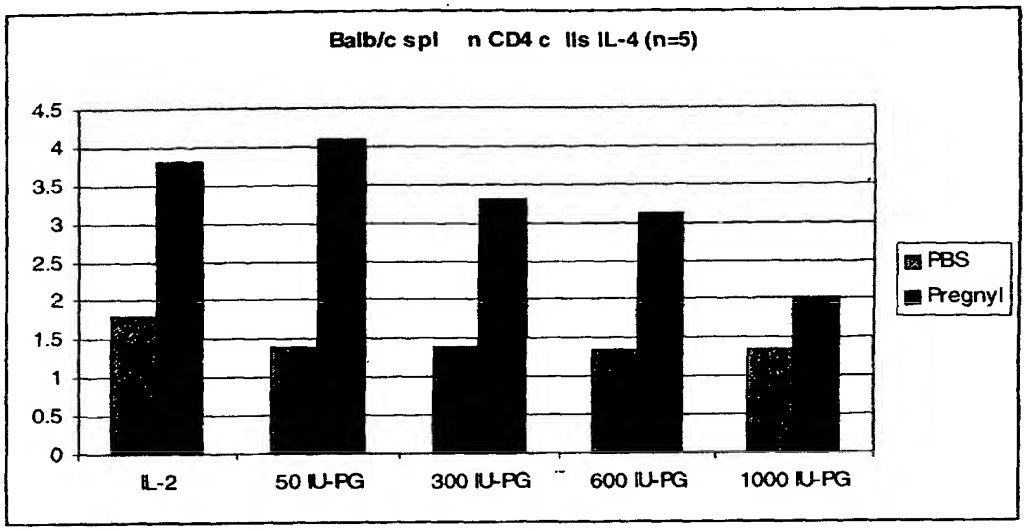

Balb/c spl n CD4 c lls IL-4 (n=5)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
IL-2
50 IU-PG
300 IU-PG
600 IU-PG
1000 IU-PG
PBS
Pregnyl

Figure 11.

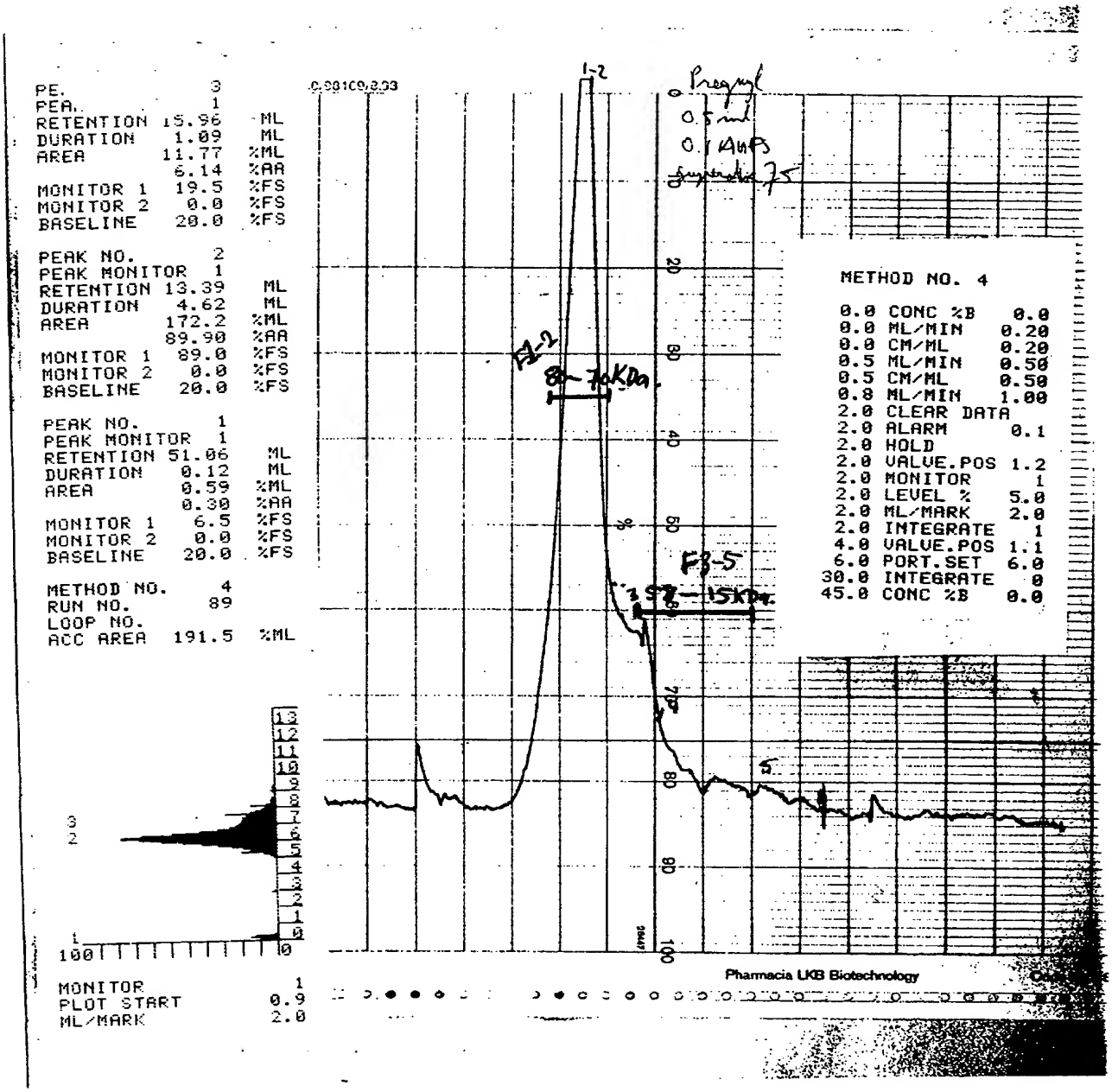

PE. 3
PEA. 1
RETENTION 15.96 ML
DURATION 1.09 ML
AREA 11.77 %ML
6.14 %AA
MONITOR 1 19.5 %FS
MONITOR 2 0.0 %FS
BASELINE 20.0 %FS
PEAK NO. 2
PEAK MONITOR 1
RETENTION 13.39 ML
DURATION 4.62 ML
AREA 172.2 %ML
89.90 %AA
MONITOR 1 89.0 %FS
MONITOR 2 0.0 %FS
BASELINE 20.0 %FS
PEAK NO. 1
PEAK MONITOR 1
RETENTION 51.06 ML
DURATION 0.12 ML
AREA 0.59 %ML
0.30 %AA
MONITOR 1 6.5 %FS
MONITOR 2 0.0 %FS
BASELINE 20.0 %FS
METHOD NO. 4
RUN NO. 89
LOOP NO.
ACC AREA 191.5 %ML
MONITOR 1
PLOT START 0.9
ML/MARK 2.0
METHOD NO. 4
0.0 CONC %B 0.0
0.0 ML/MIN 0.20
0.0 CM/ML 0.20
0.5 ML/MIN 0.50
0.5 CM/ML 0.50
0.8 ML/MIN 1.00
2.0 CLEAR DATA
2.0 ALARM 0.1
2.0 HOLD
2.0 VALVE.POS 1.2
2.0 MONITOR 1
2.0 LEVEL % 5.0
2.0 ML/MARK 2.0
2.0 INTEGRATE 1
4.0 VALVE.POS 1.1
6.0 PORT.SET 6.0
30.0 INTEGRATE 0
45.0 CONC %B 0.0
Pharmacia LKB Biotechnology

Figure 12

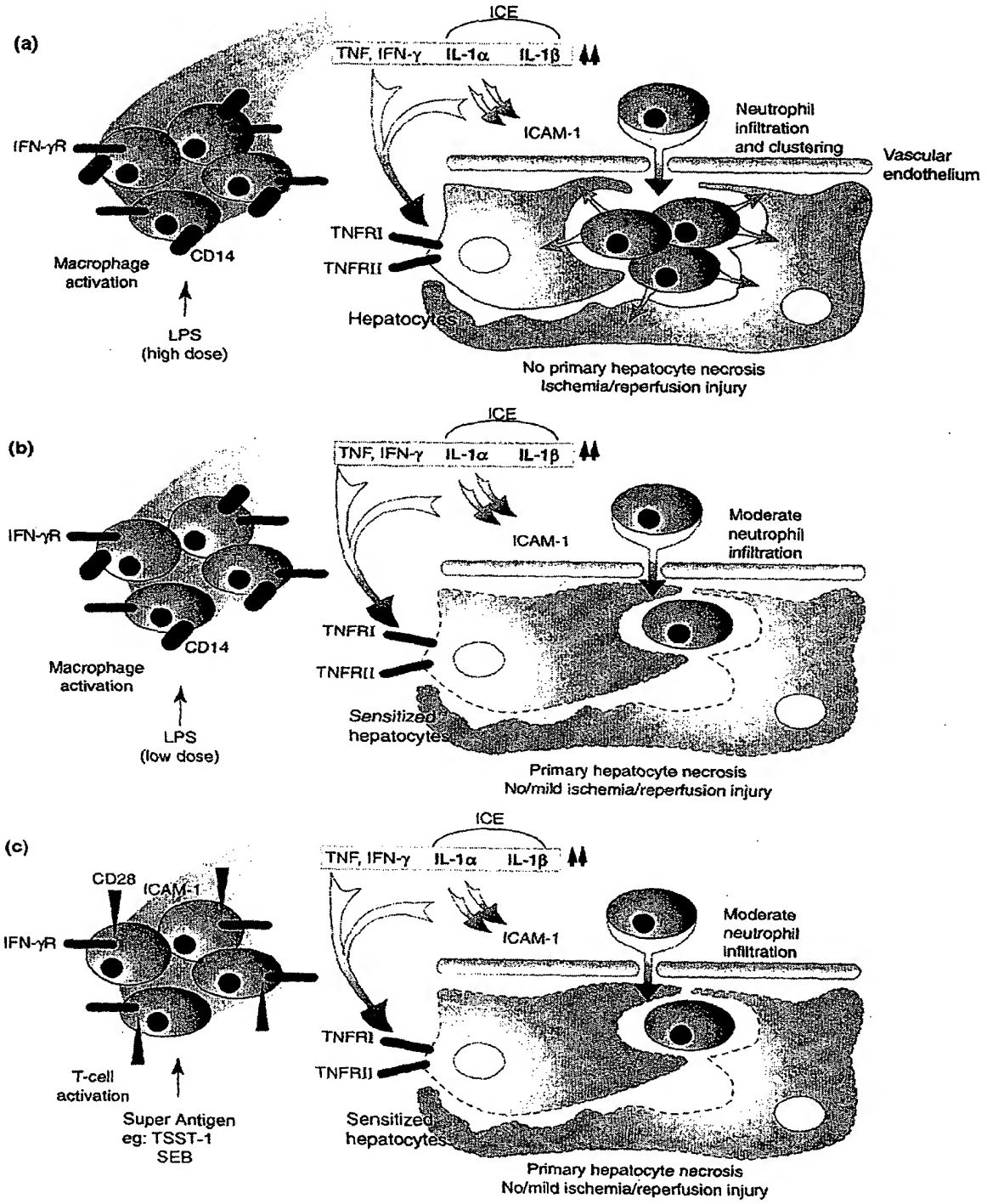

(a)
ICE
TNF, IFN-γ
IL-1α
IL-1β
IFN-γR
ICAM-1
Neutrophil infiltration and clustering
Vascular endothelium
TNFRI
TNFRII
CD14
Macrophage activation
Hepatocytes
LPS (high dose)
No primary hepatocyte necrosis Ischemia/reperfusion injury
(b)
ICE
TNF, IFN-γ
IL-1α
IL-1β
IFN-γR
ICAM-1
Moderate neutrophil infiltration
TNFRI
TNFRII
CD14
Macrophage activation
Sensitized hepatocytes
LPS (low dose)
Primary hepatocyte necrosis No/mild ischemia/reperfusion injury
(c)
ICE
TNF, IFN-γ
IL-1α
IL-1β
CD28
ICAM-1
IFN-γR
ICAM-1
Moderate neutrophil infiltration
TNFRI
TNFRII
T-cell activation
Super Antigen eg: TSST-1 SEB
Sensitized hepatocytes
Primary hepatocyte necrosis No/mild ischemia/reperfusion injury

Figure 13

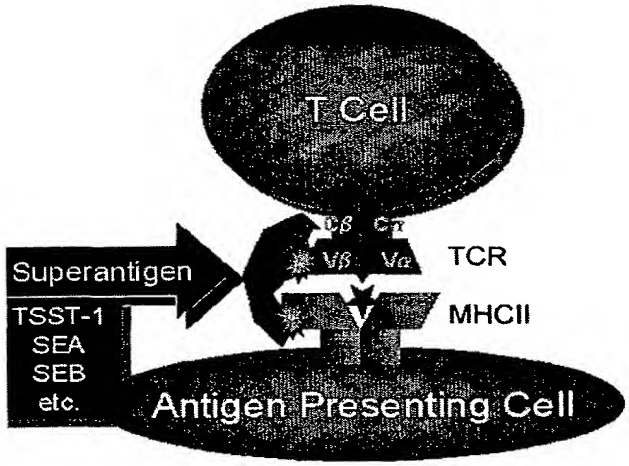
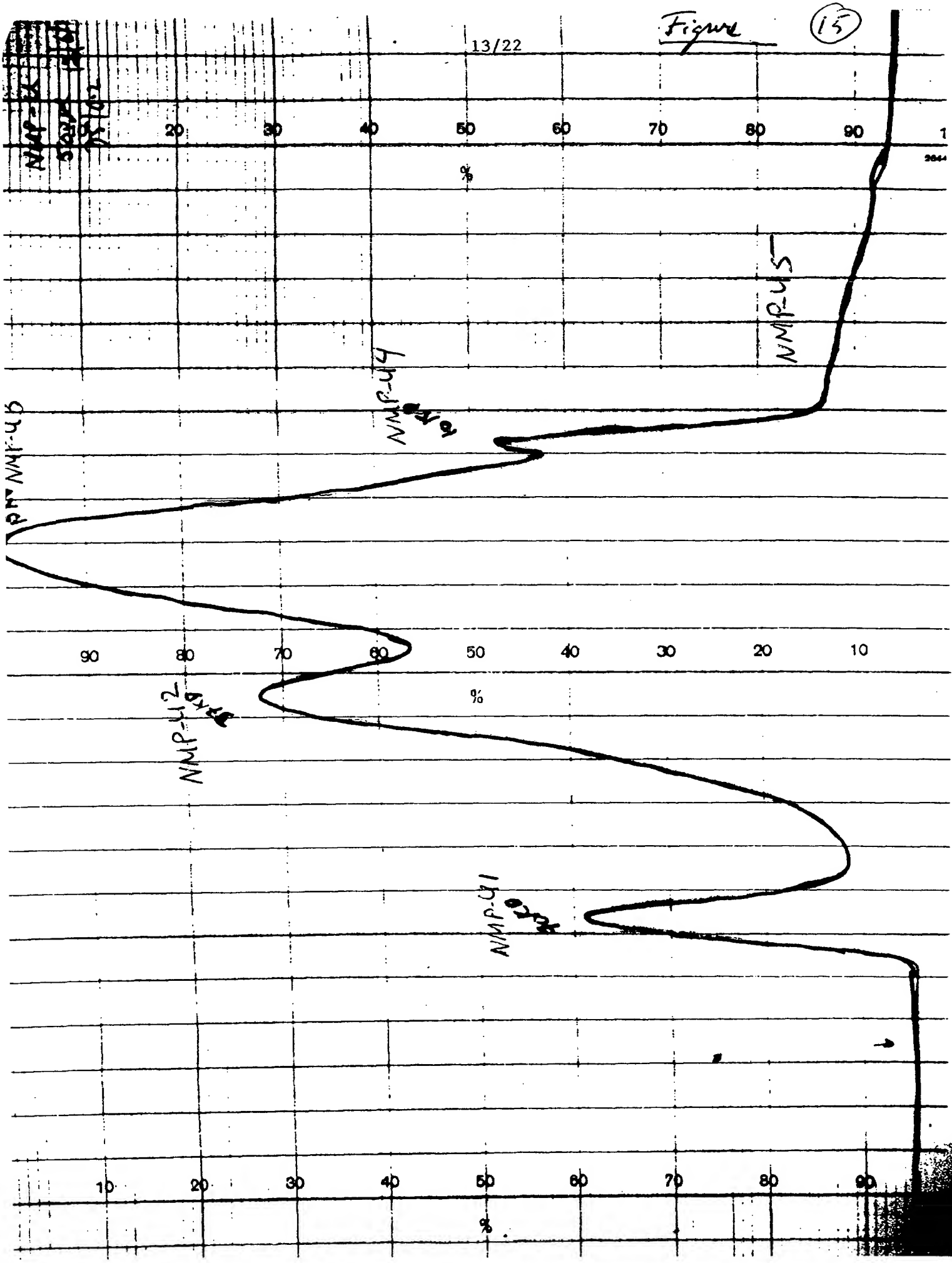

T Cell
Cβ
Cα
Vβ
Vα
TCR
Superantigen
TSST-1
SEA
SEB
etc.
MHCII
Antigen Presenting Cell

Figure 14

Figure (15)


20
30
40
50
60
70
80
90
%
NMP-45
NMP-44
NMP-43
90
80
70
60
50
40
30
20
10
%
NMP-42
NMP-41
10
20
30
40
50
60
70
80
90
%

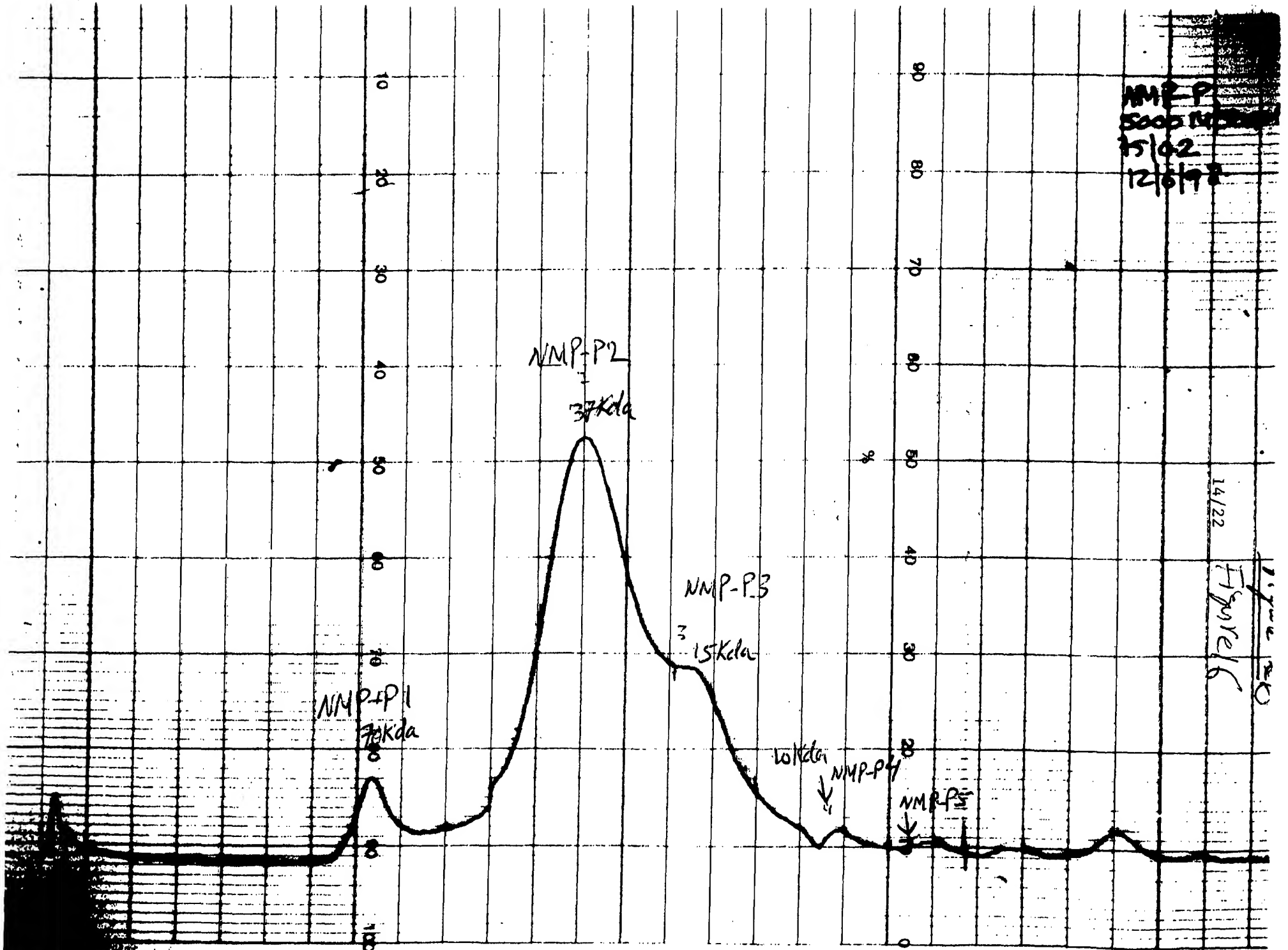
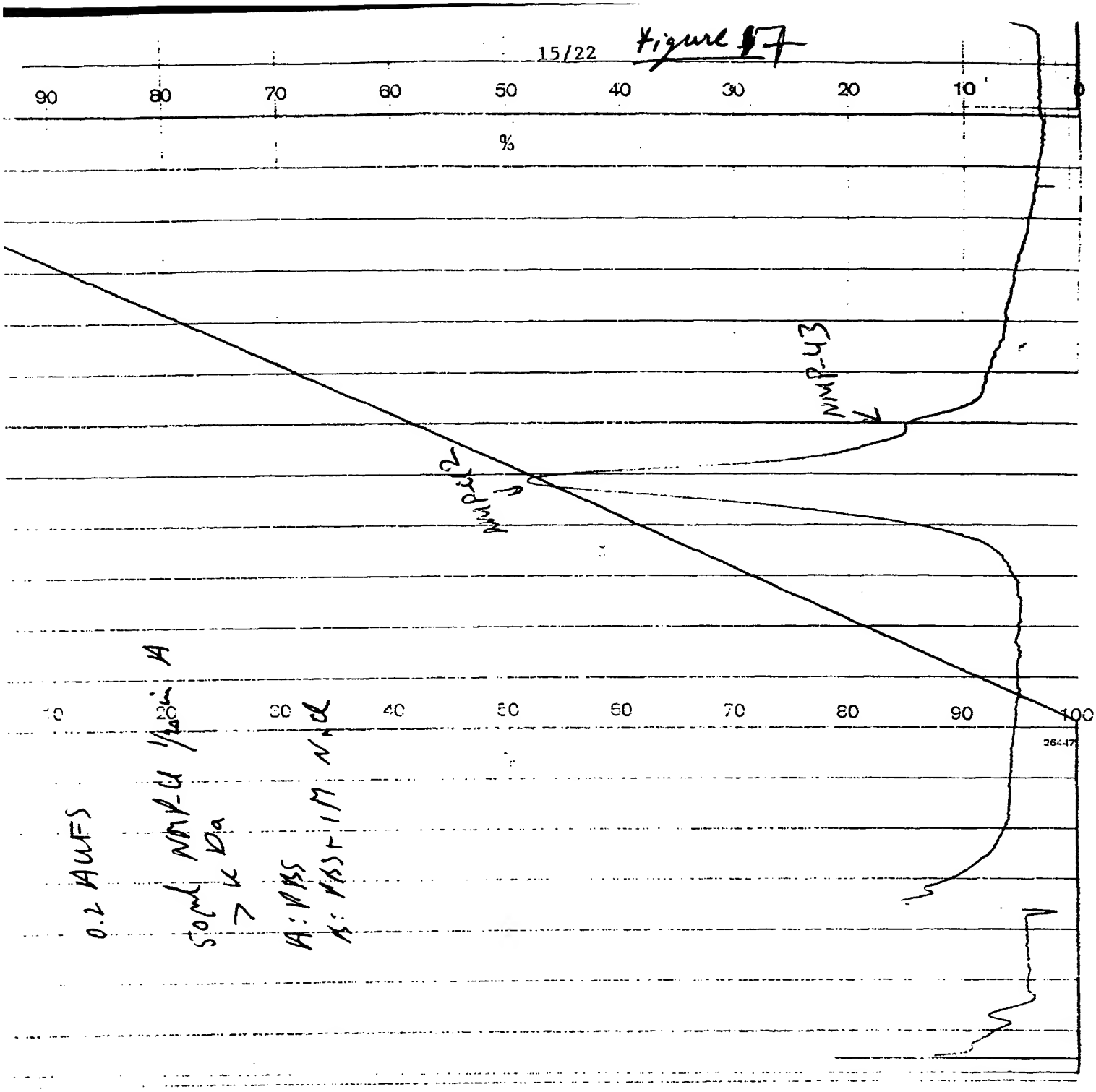
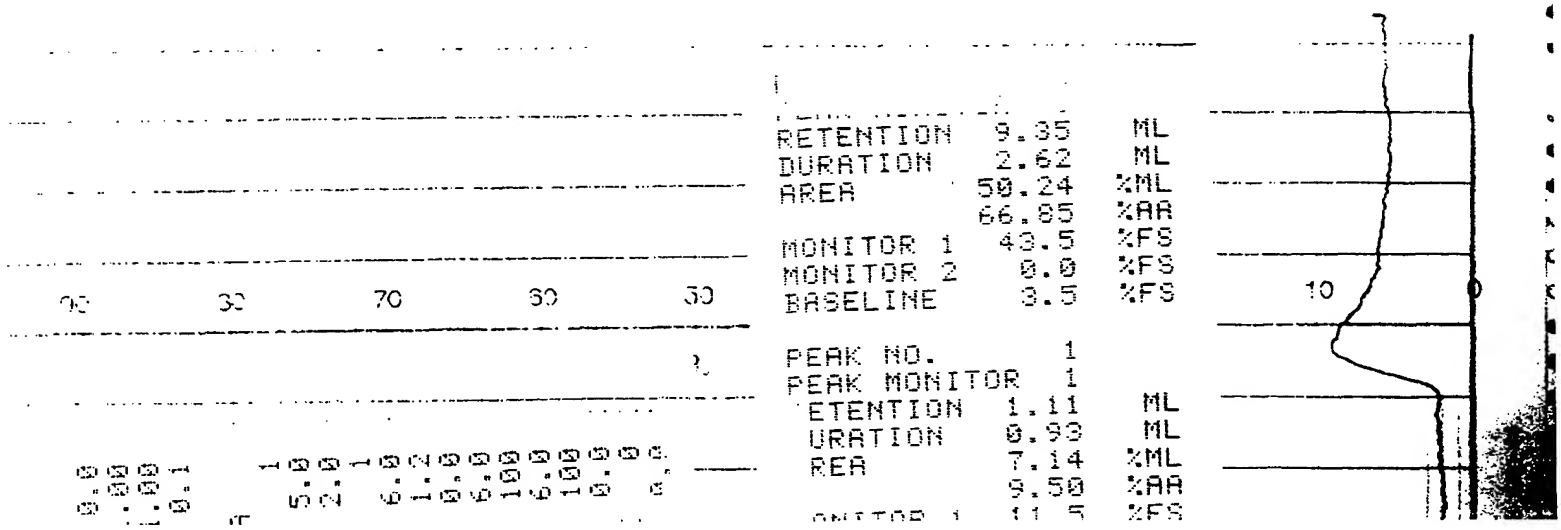


Figure 2C

Figure #7





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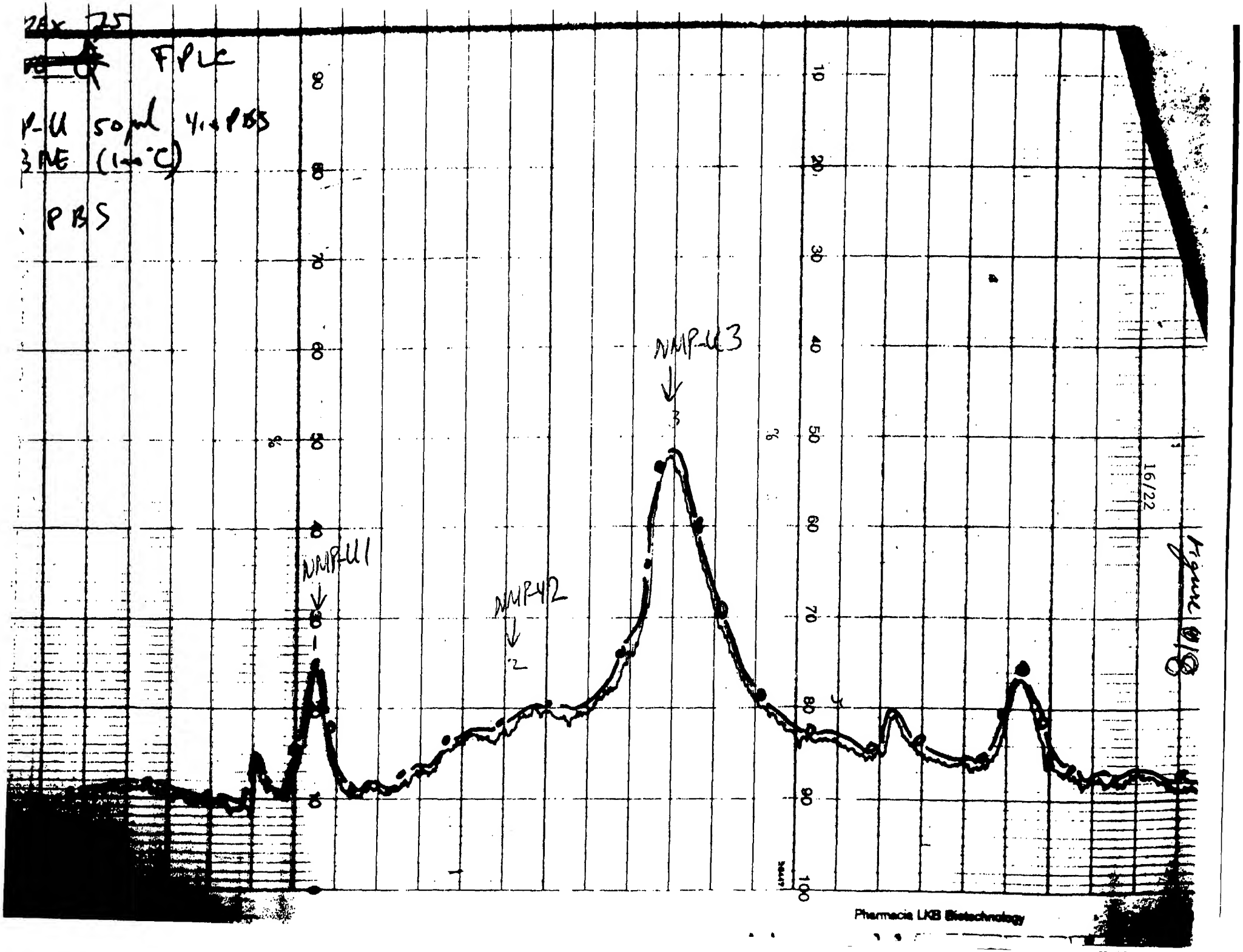
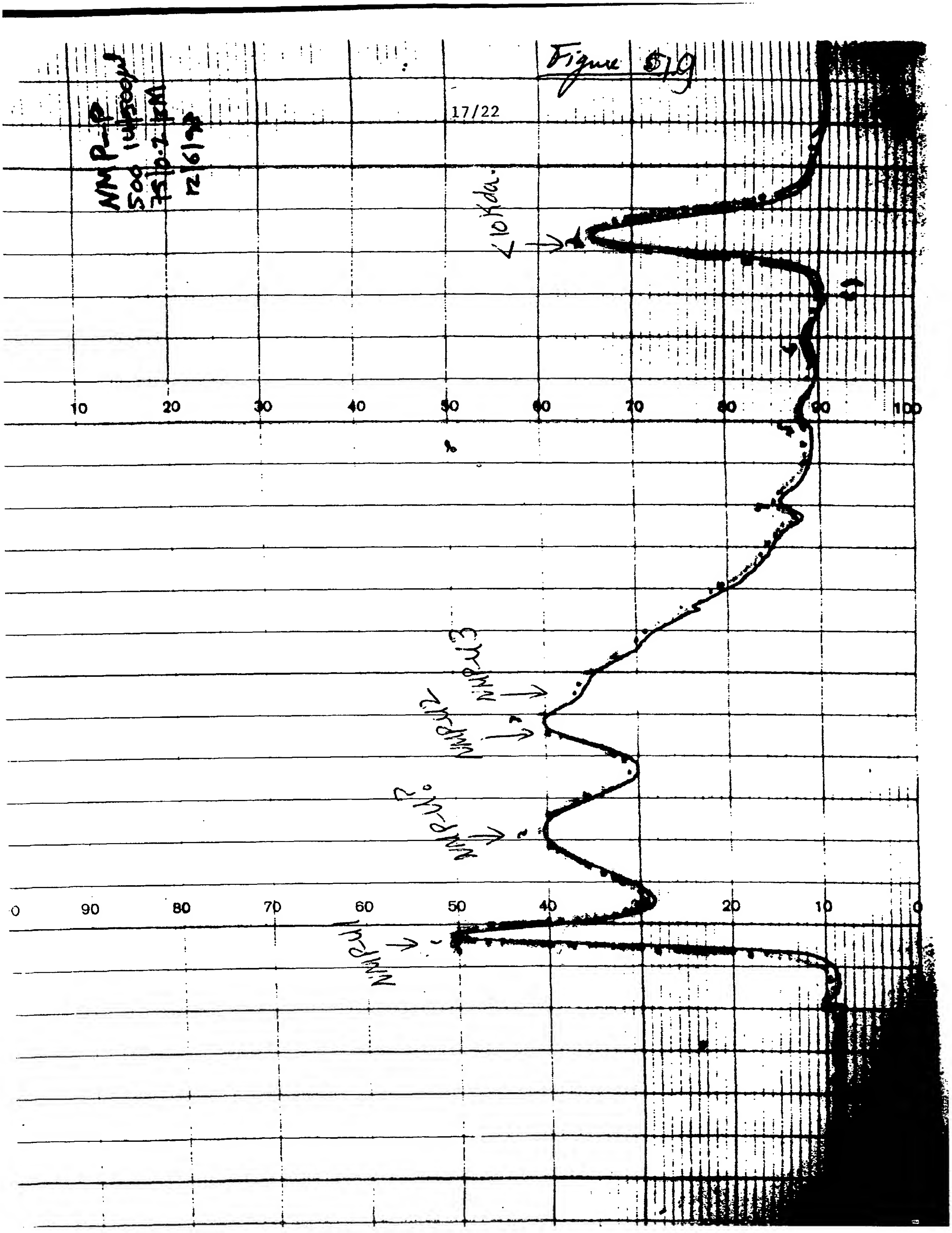

FPLC
NMP-U3
NMP-U1
NMP-U2
Pharmacia LKB Biotechnology


10
20
30
40
50
60
70
80
90
100

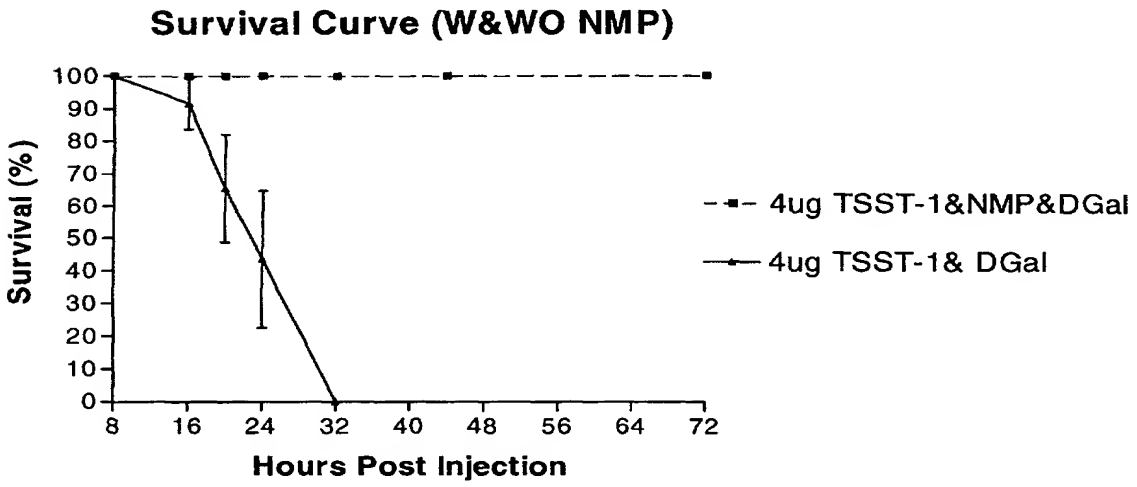

Survival Curve (W&WO NMP)
Survival (%)
100
90
80
70
60
50
40
30
20
10
0
8
16
24
32
40
48
56
64
72
Hours Post Injection
4ug TSST-1&NMP&DGal
4ug TSST-1& DGal

Figure 20

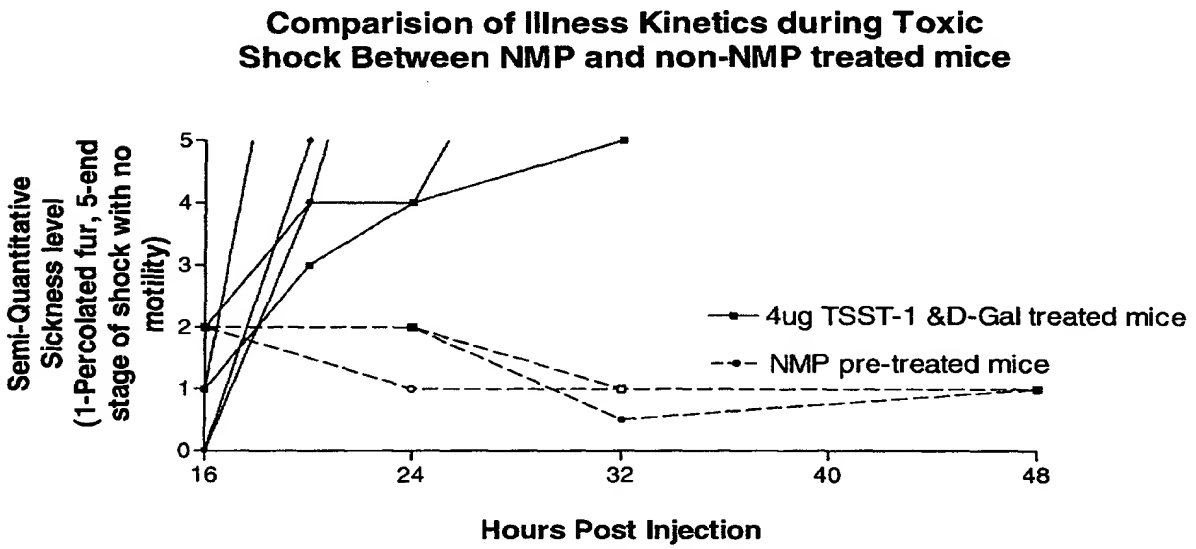

Comparision of Illness Kinetics during Toxic Shock Between NMP and non-NMP treated mice
Semi-Quantitative Sickness level (1-Percolated fur, 5-end stage of shock with no motility)
0
1
2
3
4
5
16
24
32
40
48
Hours Post Injection
4ug TSST-1 &D-Gal treated mice
NMP pre-treated mice

Figure 21

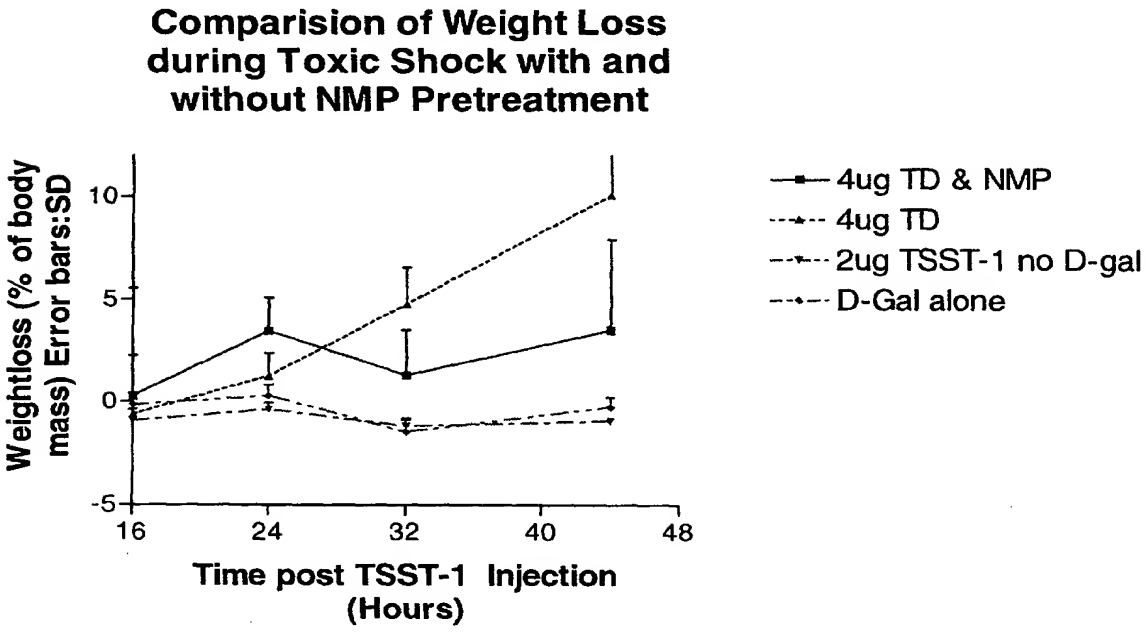

Comparision of Weight Loss during Toxic Shock with and without NMP Pretreatment
Weightloss (% of body mass) Error bars:SD
10
5
0
-5
16
24
32
40
48
Time post TSST-1 Injection (Hours)
4ug TD & NMP
4ug TD
2ug TSST-1 no D-gal
D-Gal alone

Figure 22

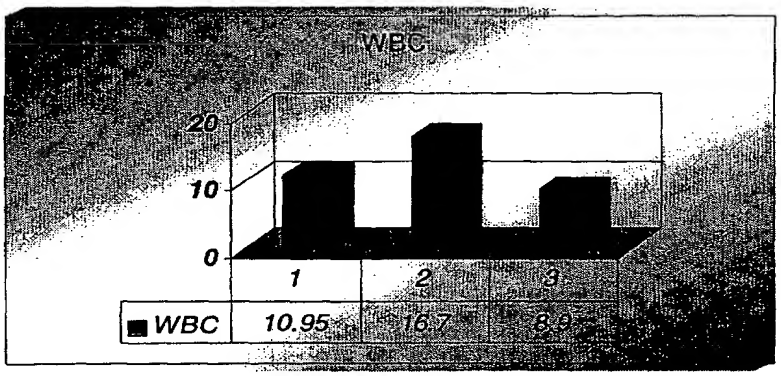


Figure 23

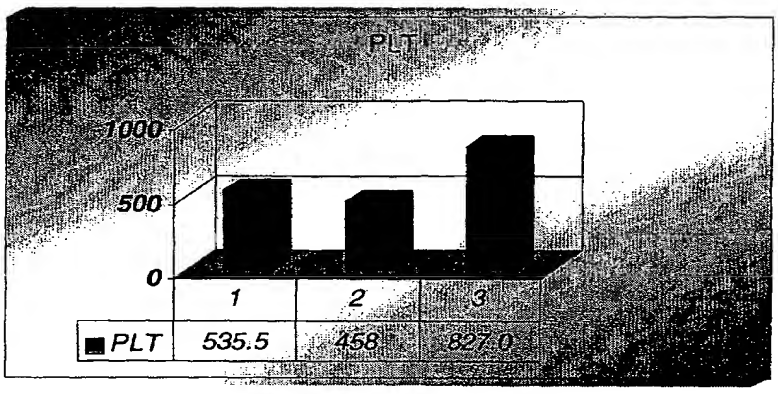


Figure 24